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THE UNIVERSITY OF ALBERTA
IRON REDUCING BACTERIA OF SOIL

by



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A THESIS
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The undersigned certify that they have read, and recommend
to the Faculty of Graduate Studies for acceptance, a thesis entitled
"Iron Reducing Bacteria of Soils" submitted by Christopher Panter
in partial fulfillment of the requirements for the degree of Master
of Science.

ABSTRACT

Techniques were developed to isolate iron reducing organisms from soil. The most effective isolates are Group 2 Bacillus and are considered to be new species. Ferric ion was shown to be utilized as a terminal electron acceptor in lieu of oxygen under anaerobiosis, and a cytochrome linked reduction system has been implicated.

Iron reducers play the dominant role in the formation of gleysols and can effectively solubilize insoluble minerals. Their numbers in soil are enhanced by increased organic matter, moisture, and alkalinity.

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I. INTRODUCTION

Iron composes roughly five percent by weight of the soil, and is a necessary micronutrient for all forms of life. Of the many agencies causing transformation of this element in soil and natural waters, that of microbial action plays a dominant role.

Iron transformation by microbes may be considered to be of two general types. The first of these is the incidental or indirect transformation, where the oxidation, reduction, precipitation, solubilization, or corrosion of iron caused by the organism is not a process of vital importance to the cell. However, as a common constituent of soil with a fair proportion in relatively free form, iron, perhaps inevitably, was to be utilized by some organisms for needs beyond that of a micronutrient. Thus the second type of transformation is a result of the use of iron directly in vital cellular processes, including the oxidation of ferrous to ferric iron by autotrophic iron bacteria as a source of energy, and the reduction of iron under anaerobiosis with the use of ferric iron as a terminal electron acceptor to regenerate the oxidative capacity of the cell.

This thesis is concerned with the reduction of ferric iron by heterotrophic microorganisms. Several theories, presented in the literature review, on the importance and mechanism of iron reduction by microorganisms in soil have been suggested by other workers. A chapter summarizing the results of research carried out in 1965-66 by F. D. Cook, M. Smit, and the author served as important background material for the thesis.

The objectives of this project were to identify and characterize iron reducing isolates, determine mechanisms of microbial reduction of

ferric iron, study the ecology of iron reducers in soil and their influence in gleization, and to determine the weathering capacity of these organisms by examination of their ability to solubilize iron-containing minerals, both primary and secondary.

II. LITERATURE REVIEW

Introduction

Microbial transformation of minerals in soil has been of interest to both soil scientists and microbiologists. Through study of the influence of microbial mineral transformations on soil fertility and soils formation processes, there has been also a revelation of new physiological processes. The classic cases include discovery of the mechanisms and importance of nitrogen and sulfur transformations in soil, and it is likely that the processes of microbial iron transformation will prove analogous and achieve like recognition.

Work on iron reduction in soils has yielded both chemical and microbiological explanations, with the more recent studies strongly indicating that iron reduction is due to the use of ferric ion by soil microorganisms as a terminal electron acceptor under anaerobic conditions, particularly during soil waterlogging.

Early Observations

Research on biological transformations of iron until 1927 was concerned primarily with oxidation and precipitation of iron by iron bacteria. However, during these early investigations some observations were made applicable to iron reduction and solubilization in soils.

Winogradsky (1888) utilized anaerobic conditions to promote solution and reduction of ferric iron as a source of ferrous iron

for his chemoautotrophic iron bacteria. Kindler (1836) noticed the solution of iron around roots in ferruginous sand. That organisms play a role in the formation of bog iron ore was an early postulation (Ehrenburg, 1836), and FeCO_3 accumulation was found in anaerobic portions of bog (Tache, 1923). Gruner (1922) showed that incubated peat extracts could solubilize iron oxides over a long term period.

Lieske (1921), who demonstrated that sucrose had a solvent effect on hydrated iron oxide, also found that molds exerted a reducing action on ferric compounds and postulated that this was due to enzymatic reduction. This conclusion was not accepted by Starkey and Halvorson (1927), who interpreted the process as being due to the fungal pellicle excluding oxygen from the medium, thereby creating a reducing condition through anaerobiosis.

Harder (1919) made the important observation that H_2S released during metabolism by microorganisms reduces ferric iron and precipitates it as FeS .

Incidental Reduction of Iron

Halvorson and Starkey (1927) were the first to consider the importance of microbial reduction of iron in soil. Equations were developed to indicate relationships in pure solution of oxygen concentration with Fe^{+++} , Fe^{++} , and H^+ . At reactions more alkaline than pH 5.0 very small amounts of Fe^{++} will occur in solution under atmospheric conditions, and even smaller amounts of Fe^{+++} are soluble.

Their primary interest was iron deposition (Starkey and Halvorson, 1927), but the processes of reduction and solubilization also were recognized as important processes in soil and natural waters. Iron transformations were considered to occur principally by chemical means. Microorganisms modify the environment by decreasing oxygen pressure in solution and promoting more acid conditions, which lead to solubilization and reduction of iron. This process is further modified by the fact that since iron in organic combination ionizes less than iron in inorganic form, high concentrations of iron beyond the inorganic solubility product of iron can exist in stable solution in soil. (In short, the reduction of iron provides no direct benefit to the cell, and hence could be considered to be a process incidental to the cell's metabolism.) Microbial attack on the organic radicals of iron can cause deposition of the iron, which could lead to the formation of bog iron and hardpan.

Mixed cultures in dextrose solution were judged to reduce iron by causing a drop in pH. While mixed cultures in a peptone medium yielded no drop in pH, there was an increase in titratable acidity which was postulated as responsible for iron reduction. Cultures grown in unsealed test tubes of medium produced less reduction and solubilization of iron oxide than cultures in tubes sealed against atmospheric oxygen. Precipitates were found more often in mixed rather than pure cultures, explained by the fact that in a mixed culture there is likely to be some organism capable of attacking the organic radical to which the iron is bound, causing instability of

the solution and deposition of the iron. Pure cultures which reduced iron included Escherichia coli and Clostridium sporogenes.

Halvorson (1930) noted that CO_2 produced by microorganisms during dissimilation of organic compounds will first increase the acidity of the solution and bring more iron from its oxide into solution. FeCO_3 will precipitate after a certain amount of iron has been solubilized. If the CO_2 pressure diminishes (by reason, for example, of a reduced level of metabolism), FeCO_3 will return into solution. Under anaerobic conditions, the consequent precipitate would be Fe(OH)_2 ; under aerobic conditions, Fe(OH)_3 . The heterotrophic microorganisms of soils were thus concluded to play a large part in iron transformations in soil.

Thiobacillus thiooxidans oxidizes sulfur to produce H_2SO_4 (Starkey, 1945), which could solubilize and reduce iron if present in sufficiently high concentration. The reverse process, sulfate reduction, also produces Fe^{++} with the reduction of Fe^{+++} by H_2S .

Early Work with Microbial Gleying

By 1940 gleying was considered to be a microbiological process. Albrecht (1940) discovered that Ca and Mg-saturated clays were more effectively gleyed than untreated or K or H-saturated clays, and postulated that since Ca and Mg were major nutrients for microorganisms, microbes were responsible for the reduction process in gleying. A waterlogging experiment with autoclaved and untreated black soils undertaken by Ignatieff (1941) showed that after initial

reduction of some of the iron by autoclaving, there was in fact oxidation in sterile waterlogged soil. Reduction of iron continued over time in the untreated, non-sterile soil. Allison and Scarseth (1942) showed that gleying was intensified by addition of a fermentable energy source such as sucrose to soil.

Reduction of Iron by Plant Extracts

Research interest in the importance of microorganisms in soil iron reduction processes was radically shifted to reduction of iron by organic compounds in soil as a result of the work of Bloomfield. Bloomfield (1950) performed gleying experiments with clays using glucose as substrate. He noted the sigmoid growth curves of glucose utilization and iron reduction, and although the final pH was too acid for field conditions, the process of gleying was interpreted as microbiological.

The next year, however (Bloomfield, 1951), he changed his opinion when the sigmoid growth curve was found with the incubation of dried grass and sterile clay. Thus either the gleying bacteria were present in the grass or the process of gleying was a side reaction of the fermentation and not in itself an intrinsically microbiological process. Sterile grass extracts were found to have considerable capacity to reduce iron and microorganisms were not required in the rate-determining step of solution of ferric oxide, since grass extracts could also solubilize iron. Although iron was dissolved, the Fe^{++} level remained rather low, attributed to the

fact that a very stable Fe^{++} -grass complex was formed which held the iron more strongly than the α - α' -dipyridal color-developing reagent. Since the extracts retained strong reducing capacity after a long length of time, Bloomfield felt that this was proof of a ferrous complex formation. Fe_2O_3 adsorbs Fe^{++} in its complex form, which when oxidized fixes new material and provides an explanation of mottling in gleys.

Therefore Bloomfield felt that there was no need to consider microbial involvement as essential in the process of gleization but rather as complementary to the action of plant leachates.

In further papers, Bloomfield (1953ab, 1954abc, 1955) dealt with podzolization. Water extracts of coniferous and deciduous leaves and bark reduced iron, forming stable ferrous and aluminum complexes. Thus sesquioxides could be mobilized in soil. These extracts, although less effective under aerobic conditions than under anaerobiosis, were not greatly so.

Schnitzer and DeLong (1955), however, found that upon electrodialysis of poplar leaf extracts enriched with iron, the major portion of the iron was present either as ferric hydroxide or in a form (possibly $\text{Fe}(\text{OH})_2^+$) readily converted to ferric hydroxide. Bloomfield (1965) reiterated that a large part of the iron in the extract-iron complex was in ferrous form, and ascribed the results of Schnitzer and DeLong to the fact that they had not worked with an anaerobic system.

In whatever form, ferrous or ferric, that iron is combined

with plant extracts, an explanation had been given for the mobilization of iron in podzolization. However, that gleization could be attributed to the reduction of iron by plant extracts proved with subsequent research to be a less satisfactory theory.

Iron Reduction as a Direct Cellular Process

Meanwhile work on the microbiological aspects of gleization continued. With Roberts (1947) there is the first inference that microbial iron reduction is not only an incidental process due to environmental and chemical changes brought about by the cell's metabolism, as Halvorson and Starkey and thought, but can be directly involved with cell respiration. An alkaline sodium-clay suspension amended with glucose was utilized as a medium for isolating iron reducing microbes from soil. Effective iron reduction by an isolate was considered to occur if the colloids turned blue-grey. An effective iron reducer which did not reduce nitrate or produce H_2S was identified as Bacillus polymyxa. Of 265 pure cultures of yeasts, actinomycetes, and bacteria, B. polymyxa was the most effective iron reducer, and a Clostridium was fairly effective. The American Type Culture Collection B. polymyxa did not, unlike the soil isolate, reduce iron.

Dilution techniques to estimate numbers of effective iron reducers in soil (presumably surface horizons) yielded 10^4 to 10^5 of these organisms per gram of soil.

Roberts discovered that the dissimilation of glucose by B. polymyxa in the presence of $Fe(OH)_3$ was more rapid and extensive than

without iron. By substituting acetate for iron, the same effect was obtained. Resting cells of B. polymyxa reduced ferric iron in a glucose, phosphate buffer, and $\text{Fe}(\text{OH})_3$ resting cell medium, but were unable to perform this reduction when glucose was omitted. Formate, molecular hydrogen, or 3-carbon metabolic intermediates were considered as the possible H-donors which reduced ferric iron. However, neither formate nor molecular hydrogen were successful substitutes for glucose in obtaining iron reduction by resting cells. Solutions (impure) of glyceraldehyde, methyl glyoxal, and dihydroxyacetone replacing glucose caused iron reduction even in the absence of cells. Although Roberts concluded that he had not found the mechanism for iron reduction, he had shown that the cell was more directly involved in iron reduction than Halvorson and Starkey had thought.

Betrémieux (1951), adapting the theory that NO_3^- and $\text{SO}_4^{=}$ were reduced by organisms under anaerobiosis in order to obtain the oxygen from these compounds, suggested that the oxides of Mn and Fe were utilized in the same manner. In a more modern context, this would be equivalent to saying that manganic and ferric ions are utilized as electron acceptors in the same manner as nitrate and sulfate.

Bromfield (1955a) found that a medium containing 0.1% yeast extract, ammonium, salts, and ferric hydroxide in which the pH did not fall below 5.5 to 6.0 was effective in demonstrating iron reduction by B. polymyxa and B. circulans. Species of

Escherichia and Aerobacter, and to a lesser extent Paracolobactrum, reduced iron best in media in which the pH during incubation fell to a low value (3.5 to 5) as, for example, in Halvorson and Starkey's glucose, salts, and ferric hydroxide medium.

Sterile clay subsoil, when amended with yeast extract medium, was gleyed when *B. circulans* was used as the inoculum but not when other organisms were used. Samples from a gleyed clay showed that a two foot depth sample was more intensively gleyed than a sample from a ten foot depth. Serial dilution in Bromfield's yeast extract medium established that the iron reducing population in the two foot sample was 10^4 per gram of soil. No iron reducers were found in the ten foot sample. Action by microorganisms was therefore felt to be supplementary to the action of plant reducing substances in gleying.

Based on the fact that Quastel et al. (1925) found that some bacteria could grow under anaerobic conditions in glycerol or lactate medium if NO_3^- was utilized as the N-source, but not if NH_4^+ was the N-source, Bromfield reasoned that *B. polymyxa* should be able to grow in glycerol and NH_4^+ if ferric oxide was supplied as the anaerobic "oxygen supply". *B. polymyxa* was shown to reduce ferric iron and nitrate when both were in the same medium under anaerobiosis, with glycerol or sucrose as the C-source. However, if NH_4^+ replaced NO_3^- , Fe^{+++} was not reduced in the glycerol medium. These results suggested that the reduction of iron is not directly involved in the oxidation of the substrate as in the case when nitrate and sulfate are reduced. (However, with NH_4^+ and Fe^{+++} in the sucrose medium, Fe^{+++}

was reduced.) Bromfield concluded that the formation of acids in the culture probably favored the solution of ferrous ion, and Fe_2O_3 was not used as an anaerobic oxygen source.

Further research (Bromfield, 1954b) demonstrated that resting cells of Bacillus circulans reduced iron in the presence of substrates, particularly malate and glucose. Neither culture filtrates nor boiled or chloroformed cells reduced iron. In a washed cell, malate, and ferric hydroxide system, the reduction of Fe^{+++} by B. circulans was stopped or severely inhibited by lack of the substrate malate, and the cell-killing treatments of chloroform, toluene, ethanol, or boiling. Cytochrome inhibitors (0.17% w/v azide and 0.17% w/v cyanide) did not inhibit iron reduction. Work with Bacillus megaterium and Aerobacter aerogenes yielded similar results. Bromfield concluded that iron reduction was accomplished by a dehydrogenase system.

Pseudomonas tralucida (Duda and Kalakutskiy, 1961) reduced iron, and iron reduction took place only when microbial cells were present. Cellular products do not induce the process.

Iron Reduction as a Microbial Process in Waterlogged Soil

Inhibitor studies have been fruitful in proving that the process of iron reduction in a waterlogged soil is microbial in nature. Sodium azide, mercuric chloride, and monoiodoacetic acid have been reported (Jamanaka and Motomura, 1959) to inhibit iron reduction in soil. Kumura et al (1963) showed that KCN (which inhibits cytochrome c), when added at a concentration of 10^{-3} M to soil immediately

after submergence, also inhibits iron reduction. There was little inhibition, however, if the cyanide was added 3 days after submergence. The reason for this could not be explained, and the masking effect that iron has on cyanide did not appear to be the cause since there was little soluble iron present after 3 days of incubation. 10^{-2} M para-aminosalicylic acid (thought to be a flavin inhibitor) and 10^{-3} M monofluoroacetic acid (to inhibit acetate metabolism) markedly inhibited iron reduction. Antimycin A (inhibits cytochrome b) had no inhibitory effect.

Two types of microbial reduction of iron in soil were distinguished by Kumura et al. The first is indirect reduction by microbial products, a process not directly involved in microbial respiration. The second type is directly involved with microbial respiration where Fe^{+++} is utilized as a terminal electron acceptor. Oxalic acid and hydroxylamine were shown to reduce iron, but by no means were these compounds as effective in reducing iron as direct microbial activity.

Succinate, acetate, and citrate substrate amendments to soil were shown by Kumura et al to have utilization curves parallel to iron reduction curves. The inhibition of iron reduction by monofluoroacetic acid, in particular, had shown an intimate relation between Fe^{+++} reduction and acetate metabolism. CO_2 production from acetate was parallel to iron reduction. Therefore ferric iron reduction is coupled to the oxidation of substrate by soil microorganisms.

Takai et al (1963b) showed that ammonification, CO₂ production, and iron reduction were parallel processes closely connected with each other. This was further proof of heterotrophic iron reduction.

The Iron Reduction Process in Waterlogged Soil and the Factors Which Influence It

The sequence of dominant microbial processes that occur when a soil has been waterlogged is best known for paddy soils. When a paddy soil is waterlogged, the oxidation-reduction potential (E_h) drops (Takai et al, 1956). As E_h drops, pH increases by as much as a unit and a half. Residual oxygen is completely utilized within one day. Nitrate reduction precedes iron reduction, the nitrates disappearing within three days. A period of rapid iron reduction occurs for anywhere between 2 days and 2 weeks, depending on the soil constituents. This is a period of ammonification and CO₂ production by facultative aerobes and sulfides, H₂, and organic acids do not accumulate during this period (Takai et al, 1963ab). Manganic compounds are reduced to manganous forms, the maximum Mn⁺⁺ concentration being reached before maximum Fe⁺⁺ concentration (Gotoh and Yamashita, 1966). Succeeding the process of iron reduction is the reduction of sulfates to sulfides, accompanied by the formation of methane. Strict anaerobes are in operation here (Takai et al, 1963a).

With drainage of the soil, E_h rises and pH drops. Fe⁺⁺ is oxidized fairly rapidly, while the Mn⁺⁺ is less subject to rapid reoxidation (Gotoh and Yamashita, 1966).

Organic matter is required for extensive iron reduction in

soils (Jamanaka and Motomura, 1959; McKeague, 1965; Daragan, 1966). Treatment of soil by adding organic matter intensifies both the iron and manganese reduction processes, increasing Fe^{++} and Mn^{++} concentration (Clark and Resnick, 1956; Gotoh and Yamashita, 1966). E_h is influenced by the amount of ferrous iron in solution. It is possible for soils almost devoid of organic matter to develop low E_h values if Fe^{++} has been released from ferrous minerals in soil, but the soil has little capacity for iron reduction without the presence of organic matter (McKeague, 1965). Intensified iron reduction by addition of organic matter to soil is observed to have a marked effect in lowering E_h and raising pH (Gotoh and Yamashita, 1966). The greatest number of iron reducing organisms are found in surface horizons with high organic matter (Daragan, 1966). Gardner (1967) showed that iron reducers were over 100 times as numerous in peat bogs as in surface horizons of mineral soils.

Due to the greater porosity of a drained paddy soil with a high content of organic matter, there is often greater opportunity for reoxidation of reduced compounds than in a soil of lower organic content. Consequently the drained soil with high organic content may show less Fe^{++} content, and greater rise in E_h and decrease in pH than in a soil with less organic matter (Gotoh and Yamashita, 1966).

The rate of decrease in E_h and length of time that rapid Fe^{++} production occurs depends not only on organic matter content, but also on texture and free iron content. Takai et al (1963a) found that a sandy loam with low free iron and low organic matter had the fastest

decrease in E_h of three submerged soils and the shortest period of rapid Fe^{++} production, which lasted only from the first to the second day after submergence. Slower than the sandy loam in rate of E_h decrease, but having rapid Fe^{++} production from the first to the sixth day after submergence, was a clay loam with high free iron and high organic matter content. A clay loam with high free iron but low organic matter reduced iron from the second to the fifteenth day after submergence, but the rate of decrease in E_h was the slowest of the three soils.

With waterlogging, the sequence of reduction processes occurs in the order from processes of high potential to low. Thus manganese reduction (with the critical potential developing the reduced ion in soil being 706 mv.) precedes iron reduction (186 mv.), which in turn precedes sulfate reduction (8 mv.). The range of E_h values observed in most studies of waterlogging range from 200 to -200 mv. On this basis Gotoh and Yamashita (1966) suggested that the redox potential in a waterlogged soil is controlled largely by the iron reduction system.

McKeague (1965) found that temperature had the effect of speeding up the gleying process ($1^{\circ}C$ to $23^{\circ}C$). Low E_h levels eventually developed in soils submerged at $1^{\circ}C$, which led McKeague to note that, as shown in some soils nearly devoid of organic matter, low E_h values are not always attributable to the action of microorganisms in the presence of organic matter. However, as discussed above, a lowered E_h value does not necessarily mean iron reduction is occurring.

Anaerobiosis is a result of waterlogging and is an important factor in increasing the number of iron reducing organisms in soil (Kalakutskiy and Duda, 1961). Except for a decrease in the fall, seasonal changes were not observed to cause major changes in the iron reducing population of soils (Daragan, 1966).

The gleying process is defined by Daragan as biological in nature, requiring the presence of organic substrate and non-specific microflora capable of reducing ferric iron. The acids formed by bacteria as they decompose organic matter contribute to the solution of insoluble free iron. Ferric iron is reduced by the bacteria to ferrous iron, which interacts with organic matter to form organo-mineral compounds in soil.

The Effect of Iron Reduction on Soil Fertility

Iron, as an essential micronutrient for plant growth, functions in the catalase, peroxidase, and cytochrome respiratory systems (Tisdale and Nelson, 1966), acts as an essential coenzyme in the formation of chlorophyll, and is a component of ferridoxin.

Halvorson and Starkey (1927) felt that highly alkaline conditions cause iron to be immobilized in ferric form, thus creating scarcity of iron for plants. Highly acid conditions, on the other hand, lead to toxic quantities of Fe^{++} in the soil. Kliman's study (1937) demonstrated that plants take in iron as Fe^{++} . Reduction of Fe^{+++} before entry into the plant was considered to be brought about by the action of soil organic matter, microorganisms, or

reducing substances always present around the plant root. Iron is returned to the soil as ferrous organic residues which by microbial degradation release Fe^{++} . In essence, therefore, Kliman postulated an iron cycle in the soil.

Modern concepts (Tisdale and Nelson, 1966) state that iron may be absorbed by plant roots in both oxidation states or in the form of complex organic salts and chelates. However, the metabolically active form of iron appears to be Fe^{++} , and plant tissues containing large quantities of Fe^{+++} may show deficiency symptoms.

Japanese workers have established that with waterlogging of soil and iron reduction, soil E_h drops and pH increases. With sulfide production, the Fe^{++} produced prior to sulfur reduction is important in combining with the $\text{S}^{=}$ of H_2S to form an insoluble FeS precipitate, thereby preserving iron and sulfur nutrients from leaching from the soil and reducing the harmful effect of both Fe^{++} and $\text{S}^{=}$ on rice plants. Heavy compost applications to fields that are to be waterlogged will cause intensified iron and sulfur reduction, but although there is initial inhibition of young rice plants, the result is a higher yield. The rice root acts not as a reducing zone but as an oxidizing zone, thereby helping to ameliorate Fe^{++} poisoning (Gotoh and Yamashita, 1966).

Utilization of Biological Reduction of Iron in Laboratory Procedures

Allison and Scarseth (1942) attempted to find a practical use for iron reducing microbes by utilizing a biological reduction method for removing iron oxides from clays. Sucrose was added as substrate

and allowed to ferment, and the effluent with the Fe^{++} was removed and tested. Compared to the Truog H_2S chemical method, the biological method was as effective in removing iron, yet degraded Al and Si components of the clays to a lesser degree. The disadvantage was the time required for the fermentation.

Bromfield and Williams (1963) improved this biological reduction method in order to estimate the free iron content of soil. The soil was pretreated with H_2O_2 to disperse soil particles, release organically-bound iron, and increase the solvent action of the organic acids formed. A mixed iron reducing culture was used as inoculum, and the biological reduction process was hastened by incubating the culture at 35°C . Their conclusion was that although the method was effective for estimating free iron, it was too cumbersome in comparison with the equally effective oxalate chemical method.

Reduction of Other Minerals by Microbes

Both respiratory and assimilative nitrate and sulfate reductions by microorganisms are familiar phenomena. Microbial phosphate reduction is known to occur and attention is being given to its role in soil (Alexander, 1961). The foregoing discussion has indicated that microbial reduction of iron is also an important process in soil.

The geochemistry of iron and manganese is closely related and there is similarity of properties of some of their compounds. Mn in MnO_2 acts as a terminal electron acceptor in animal tissue and yeasts (Silverman and Ehrlich, 1964). Trimble and Ehrlich (1968) have concluded recently that manganese reduction is a respiratory process.

in a marine Bacillus and a coccus.

McCready et al (1965) showed that Salmonella heidelberg reduces selenite to selenium in what is apparently a detoxification mechanism. Woolfolk and Whitely (1962) demonstrated that compounds of the following elements are reduced as hydrogen is oxidized by cell extracts of Micrococcus lactilyticus in a hydrogen atmosphere: arsenic, bismuth, selenium, tellurium, lead, thallium, vanadium, manganese, iron, copper, molybdenum, tungsten, osmium, ruthenium, gold, silver, and uranium. Many of these reductions could be linked to dehydrogenase systems. The importance and extent of microbial reduction of these and other elements (such as zinc) in soils has yet to be investigated.

Conclusion

A number of mechanisms by which microbes transform iron have been suggested. Oxidation of Fe^{++} to Fe^{+++} may be carried out by chemoautotrophs, facultative chemoautotrophs, or heterotrophs. Precipitation of iron in soils can occur with the release of O_2 by algae, the increase in pH caused by proteolytic organisms, or with microbial attack on the organic portion of organic iron compounds (Alexander, 1961).

Microbial transformations of sulfur can alter the status of iron in the soil. Sulfur oxidizing bacteria form H_2SO_4 , which in high enough concentration can solubilize and reduce iron. Sulfate reducers and heterotrophs which attack S-containing proteins release

H₂S, which reduces ferric ion to form FeS precipitate. Sulfate reducing bacteria have also been implicated in the corrosion of iron metal in soil.

Conditions in soil which have been considered favorable to the reduction of iron include anaerobic conditions, a pH lower than 5, or a redox potential lower than 200 mv. Waterlogging enhances reducing conditions. Iron reduction and solubilization can occur by means of the production of acids during soil metabolic processes, by depletion of the O₂ supply by microorganisms and roots, by production of reduced compounds which lower the redox potential, and by the action of plant extracts.

Other heterotrophic bacteria bring about reduction of iron enzymatically. Soil incubation experiments indicate that Fe⁺⁺⁺ is utilized as a terminal electron acceptor, and that this mechanism is the most important process in gleization. However, the mechanism has yet to be demonstrated unequivocally with pure cultures of iron reducing organisms.

III. BACKGROUND

This chapter is a brief composite report of the work done by Dr. F. D. Cook, M. Smit, and the author in 1965-66 which served as essential background for the thesis. The project was initiated to determine whether iron reducing bacteria utilized ferric ion as a terminal electron acceptor in lieu of oxygen under anaerobic conditions, a question never fully resolved in the literature to this date.

The first consideration was the selection of a medium for isolating effective iron reducing organisms. Medium B, which consisted of salts, $(\text{NH}_4)_2\text{SO}_4$, and 1% ferric ammonium citrate, was more effective in isolating a wide variety of the heterotrophic iron reducers when transformed into Medium B3 by addition of 0.5% yeast extract. (Exact composition of media is supplied in Appendix I). B7 medium, a rich nutritional medium which added further micronutrients and substituted 0.5% peptone in place of the $(\text{NH}_4)_2\text{SO}_4$ in B3, was much superior. Later an equally effective medium for isolation, B10, was utilized. In this medium the ferric ammonium citrate of B7 was replaced by ferric phosphate.

An effective isolation procedure for iron reducers was begun by the inoculation of 0.1 g. of soil in test tubes of B7 or B10 medium. Reduction was usually complete within three days at room temperature and ascertained by a spot test for ferrous ion with 2% o-phenanthroline or α - α' -dipyridal. Equally satisfactory was simple observation of a change in color of the medium from brown to

green, decolorization with formation of a white precipitate, or formation of a green, green-black, or black precipitate which was often gelatinous in nature. Usually these first cultures were enriched by transfer to fresh iron broth and after reduction purified by successive passage on aerobic iron medium plate. Single colonies of the resulting pure cultures were placed back in iron broth to verify reduction capability.

Variations of this isolation procedure have proved useful. Pasteurization of the inoculated sample aids in obtaining some of the most effective isolates, but reliance cannot be placed entirely on this technique since not all of the sporeforming isolates can germinate in iron medium. Sealing of iron broth tubes with 2% agar caps or placing the tubes in an anaerobic chamber can eliminate many non-iron reducing competitors in the isolation medium. Attempts to isolate iron reducers from mixed cultures on iron plates under anaerobiosis by picking colonies with marked green areas surrounding them has not been successful since reduction of iron can occur chemically in agar with prolonged anaerobiosis. A useful headmark to distinguish some of the iron reducing colonies in a mixed culture on aerobic iron agar plate is a characteristic depression of the agar in the centre of the colony.

Isolates obtained by these methods have variable capacity for reducing iron, from weak to very strong. Most of the effective reducers are Bacillus sp.

Isolate 92 easily established itself as our most effective

iron reducer. This Bacillus, isolated by M. Smit after pasteurization of a lake bottom mud sample, completely reduces the ferric iron present in a tube of B7 or B10 broth. On iron plate 92 grows as a translucent colony which markedly depresses the agar, while colonies on plate count are raised and cream-colored. Morphologically 92 is a rod with a spore causing terminal swelling. In Vitamin B12 broth (denitrifier medium) 92 reduces NO_3^- to NO_2^- . Under anaerobiosis in B7 or B10 iron broths, 92 shows good growth and reduction of ferric iron while there is never more than slight visible growth in control media A1 and A3 which have only micronutrient quantities of iron. This suggested that Fe^{+++} is utilized by 92 as an electron acceptor under anaerobiosis, an aspect covered in greater detail in the thesis.

Classification of some of the Bacillus iron reducers, including isolates 92, 130, 1-4, and 8-2, according to Bergey's Manual (Breed et al, 1957) and Smith et al (1952) revealed that none of these isolates were found to fit the descriptions of any of the Bacillus species listed and suggested that new species had been found.

Further work was carried out on the nutritional requirements of iron reducing bacteria. When yeast extract of B7 medium was reduced from 0.5% to 0.3% and $(\text{NH}_4)_2\text{SO}_4$ was substituted for peptone, fair response was obtained from most isolates. Addition of casamino acids or sucrose to this new medium gave no benefit. However, the addition of further yeast extract improved growth and reduction. When yeast extract in B10 medium was cut from 0.5% to 0.3% and peptone was omitted with no $(\text{NH}_4)_2\text{SO}_4$ added to replace the loss of this N-source,

the results were at least as good as for the abbreviated B7 medium, indicating that for most of these isolates mineral NH_4^+ was of little benefit. However, both of these abbreviated media, even with the extra yeast extract improving growth and reduction, were inferior to B7 or B10.

Basal medium B with iron and salts but no energy source other than the citrate in ferric ammonium citrate were tested with various amendments with Bacillus 92. Neither trypticase or peptone alone were satisfactory additions for growth. Yeast extract additions gave good response and yeast extract plus peptone yielded maximum growth and reduction.

One class of iron reducers which produced gas bubbles beneath agar caps of sealed B7 (ferric ammonium citrate) tubes were shown to be citrate fermenters. These organisms grew as well in sealed A1 control tubes as in B7, and the reduction of Fe^{+++} thus appeared to be incidental to their growth under anaerobic conditions rather than obligately required. Since obligate reducers were of primary interest and the citrate fermenters had a tendency to overrun primary isolation cultures, B10 (ferric phosphate) was utilized as the routine medium.

Several of the sporeforming iron reducers, including Bacillus 92, failed to grow after pasteurization at 80°C for 10 minutes after inoculation into B7 or B10 broth, despite the presence of spores in stained preparations of these pasteurized cultures. However, they would usually grow if previously cultured in Vitamin B12 broth,

transferred to fresh Vitamin B12, and pasteurized. The problem appeared not to be in spore formation but germination. Addition of Vitamin B12 broth ingredients KNO_3 or Vitamin B12 to iron broth did not aid germination, nor did growth occur after pasteurization when iron concentration in B7 was reduced. In fact, there was no growth after pasteurization in A1 control medium, which is a good medium for growth under aerobic conditions. Thus the high iron concentration of the iron broths did not appear to be inhibitory to germination and the question remained unresolved.

Some isolates reduce both iron and nitrate. A few iron reducers are, in fact, denitrifiers. Bacillus 92, a nitrate reducer, and Bacillus 130, a non-nitrate reducer, were inoculated into tubes of B10 and B10 + KNO_3 , 130 reduced Fe^{+++} but not NO_3^- ; 92 reduced NO_3^- but did not proceed to reduce Fe^{+++} . NO_3^- was concluded to be the preferred terminal electron acceptor under anaerobiosis for 92. Since NO_3^- did not hamper the reduction of Fe^{+++} by 120, it appeared that NO_2^- inhibited Fe^{+++} reduction by 92.

This summary presents only a portion of the work done in 1965-66. Other work initiated was carried on as part of the thesis work, and is discussed, therefore, in greater detail in the thesis body.

IV MATERIALS AND METHODS

Identification and Characterization of Iron Reducing Isolates

Purification and Selection of Type Isolates

Over 200 iron reducing cultures were isolated from soils, lake muds, and peats by the methods outlined in Chapter III. Since many of these were mixed cultures, the first task was the purification of these cultures by successive aerobic plating for single colonies on B10 iron medium plates. Iron reducing capability of these purified isolates was verified by visual observation of strong reduction upon transfer to B10 iron broth. (B10 medium is composed of salts, 0.5% peptone, 0.5% yeast extract, and 0.47% ferric phosphate. The pH is adjusted to 7.0 with 1 N NaOH. Detailed composition of all media utilized during this project is supplied in Appendix I.)

In order to reduce the resulting five dozen pure cultures to a manageable number, type isolates were selected after comparing their colonial morphologies on B10 plate, the rapidity with which they reduced tubes of B10 broth, the nature of precipitate, if any, formed in B10 broth, and other characteristics which had been observed during work with these cultures.

A list of the isolates and their source is shown in Table 1. Bacillus circulans (henceforth designated in tables as B. circ.), B. cereus var. mycoides (B. myc.), and B. subtilis (B. subt.) proved to be poor iron reducers but were useful for purposes of comparison since they were Bacillus whose taxonomy was known. I₂, a denitrifier

Table 1. Iron reducing isolates selected for study and their source

Isolate	Source
1-3	lawn soil (Edmonton)
1-4	" " "
1-6-2	" " "
1-8	" " "
1-10	" " "
1-12-2	" " "
3	C horizon of Dark Grey Wooded soil (Carvel series)
6	B and C horizons of Dark Grey Wooded soil (Uncas series)
7-2	creebank (Whitemud Creek)
8-2	creebank (Whitemud Creek)
12-4	riverbank (North Saskatchewan River, Edmonton)
14-4	decomposed iron nodule from C horizon of Orthic Black Chernozem (near Ellerslie)
19	Bm horizon (19" depth) of Orthic Black Chernozem (Angus Ridge series)
22	Ah horizon (14" depth) of Orthic Black Chernozem (Angus Ridge series)
92	lake bottom mud
130	lake bottom mud
627	buried soil at a buffalo jump (near Calgary)
629-3	" " " " " " "
633	" " " " " " "
634	" " " " " " "
635	" " " " " " "
636	" " " " " " "

Isolate	Source
I ₂	from an Edinburgh collection
<u>B.circ.</u>	culture collection of the Alberta Provincial Laboratory (Edmonton)
<u>B.myc.</u>	used as food source for a myxobacter sent from an East German collection
<u>B.subt.</u>	from Department of Microbiology (University of Alberta, Edmonton)

from an Edinburgh collection, and the 600 series, comprising denitrifiers and nitrate reducers from buried soils of a buffalo jump near Calgary, were obtained from Dr. F. D. Cook's collection. 92 and 130 were isolated from Alberta lake bottom muds. The remainder were isolated from various soils by the author and are designated by source number.

Routine Procedure

A few points of routine procedure utilized in this thesis should be noted. Stock cultures of the selected isolates were maintained in unsealed tubes of B10 broth and usually transferred to fresh B10 every two weeks. These stock cultures served as the source of inocula for all experiments unless stated otherwise. Periodic checks for purity of these stock cultures were made by plating them for single colony on B10 plate.

By use of the term "unsealed tube", it is meant that although the inoculated tube of medium was, of course, capped to maintain a pure culture, access of air to the medium was not prevented. The

6 ml. of medium utilized for each 1 x 15 or 1.1 x 1.2 cm. test tube resulted in a depth of medium sufficient to provide conditions anaerobic enough for iron reduction to take place, and unless specified otherwise, this was the general condition for most experiments. Also, except in a few experiments, all incubations were at room temperature.

Identification of Type Isolates

All of the selected type isolates were capable of aerobic growth. In order to determine whether they were facultatively anaerobic, the isolates were inoculated into 6 ml. of B10 broth in unsealed tubes and incubated under anaerobic conditions in a large dessicator. The dessicator was filled and evacuated three times with commercial grade nitrogen. Provision of CO_2 and elimination of residual O_2 was accomplished by use of the iron wool method for anaerobiosis (Parker, 1955). Growth was examined after one week.

Month old iron reducing cultures in B10 broth were transferred in duplicate to fresh B10 broth and pasteurized at 80°C . for 10 minutes. Since some of the known sporeformers (e.g. isolate 92) failed to show growth, other media were utilized. Three week B10 cultures were transferred into duplicate sets of B10, A3 (the control medium for B10, containing only micronutrient quantities of iron), and Vitamin B12 broth (a routine medium for denitrifiers and nitrate reducers). One set of each inoculated medium was pasteurized and the other left as an unpasteurized control.

Phase contrast examination on a Wild microscope was made of all cultures for cell morphology, motility, and spores. Phase microscopy was found to be superior to the Bartholomew and Mittwer "cold" stain (Conn et al, 1957) for examination of spores. All cultures were examined at various ages (from one day to six weeks) in B10 and A3 broths and on plate count agar and B10 plate. Where description of spores was deemed reliable, the organism was classified as a Group 1, 2, or 3 Bacillus according to the key in Smith et al (1952).

Characterization of Type Isolates

Type isolates were plated for single colonies on B10 plate. (B10 plate is simply B10 broth solidified with 2% agar. 1.5% agar, the agar concentration usually used to solidify most media, results with the high iron concentration of B10 in a surface which is too easily damaged by streaking.) Single colonies were described after three days of aerobic incubation at room temperature. This procedure was repeated three times to ensure that the colonial characteristics described were stable.

Several simple carbohydrates were tested to determine whether they could be utilized as the sole energy source by the isolates for growth and reduction of iron under anaerobiosis. This was done not only to characterize the isolates but also as an initial step in determining the composition of media for the resting cell experiments to follow.

Essentially the basic medium, B19, for this nutritional experiment was an abbreviated B10 medium in that the salt and iron content

remained the same, 1% $(\text{NH}_4)_2\text{SO}_4$ was substituted for peptone, and yeast extract was cut from 0.5% to a growth factor level of 0.01%. Sterile sets of 8 ml. screw-cap tubes of B19 were prepared and to each tube of a set one of the following sterile solutions was added: 0.6 ml. 5% Na-pyruvate, 0.3 ml. 10% Na-succinate, 0.3 ml. 10% sucrose, 0.3 ml. 10% Na-lactate, 0.3 ml. 10% dextrose, 0.3 ml. 10% Na-acetate, and 0.3 ml. 10% Na-citrate. Isolates were inoculated into all of the amended media, uninoculated controls were left for each, and all tubes were aseptically filled completely with B19 and sealed. Five days of incubation were allowed. Any isolate which showed, by visual inspection, growth and reduction in these media were retested.

Ability of these isolates to denitrify or reduce nitrate to nitrite was determined by growing them for four days in duplicate in agar-capped tubes of Vitamin B12 broth, a routine medium for this purpose. Nitrate reduction was ascertained by spot test with diphenylamine and Tromsdorf reagents, and denitrification was verified by absence of NO_3^- and NO_2^- , gas bubbles under the agar cap, and reaction greater than pH 7.6 as judged by a Brom-Thymol Blue spot test.

Sulphate reduction to sulphide by isolates was tested by inoculation into unsealed tubes of routine Butlin's medium for sulphate reducers. 6 ml. of Butlin's medium plus 2 drops of sterile 10% Na_2SO_3 solution per tube and 6 ml. of Butlin's medium (minus sulphate) plus 2 drops of sterile 10% $\text{Na}_2\text{S}_2\text{O}_3$ per tube were utilized to determine, respectively, sulphite and thiosulphate reduction to

sulphide. A positive reaction for all three tests consists of a black precipitate of FeS . Since none of the isolates proved to be sulphate reducers, and only two were sulphite reducers, the possibility that Butlin's medium was nutritionally inadequate was checked. 0.35% (w/v) Na_2SO_4 and Na_2SO_3 were added, respectively, to 2 batches of B10 minus FePO_4 to form S_1 and S_2 media, which were dispensed into tubes containing a clean iron nail and used to test all isolates.

The iron reducers were grown in unsealed tubes of nutrient broth and checked after four days visually for growth and by spot test with Nessler's reagent for NH_4^+ production. Also tested for evidence of mineralization of organic nitrogen were one week and two week cultures in B10 broth.

Isolates were inoculated into B10 broth, B10 broth amended with 1 $\times$ /1. Vitamin B12, and Vitamin B12 broth. Sets of inoculated media were incubated at room temperature, 34°C , 50°C , and 65°C . and examined for growth and iron and nitrate reduction after 5 days. With this experiment some idea of the temperature tolerance of these organisms could be obtained and it could be determined whether Vitamin B12 was required for iron reduction at high temperatures, as it appears to be for thermophilic nitrate reduction (Cook, 1968).

Visual observation was made of the time required for strong reduction to appear in B10 broth after inoculation by isolates from B10 cultures of various ages (one day to one month) in order to determine whether this would aid in distinguishing between cultures.

Appearance of precipitates in B10 cultures was also noted.

The precipitate in a one week culture of isolate 92 was analyzed by X-ray diffraction by Mr. S. Wiskell of the Alberta Research Council.

The Mechanism of Microbial Iron Reduction

A Method for the Determination of Fe^{++} in Cultures

The method utilized for the determination of Fe^{++} was essentially the orthophenanthroline method of Krishna Murti et al (1966) for the determination of Fe^{++} and Fe^{+++} , except that the step of utilizing hydroxylamine hydrochloride to reduce all iron present in solution was omitted except when preparing the standard curves (Appendix II). A further modification was the preparation of standard curves in the appropriate dilution of the iron-free control medium for the medium being tested. For example, if Fe^{++} content of B10 medium was to be determined, the standard curve would be run utilizing A3 medium.

One problem which appeared with this method for determination of Fe^{++} was not the expected one of oxidation but rather autoreduction of Fe^{+++} if the test solution was allowed to stand in the pH 3.5 Na-acetate-acetic acid buffer for a long length of time. In order to determine what length of time the o-phenanthroline color developing reaction should be allowed to proceed, the optical densities of several tested samples were followed over time at 505 m μ on a Bausch and Lomb Spectronic 20. The samples utilized were 4 day cultures of isolates 92, 130, 14-4, and B. mycoides and several batches of B10 medium.

Incidental Reduction of Iron

The following experiments were carried out to determine whether iron reduction was incidental to the metabolism of the cell. In other words, it was known that the isolates caused the reduction of iron since uninoculated controls do not reduce, but the question was whether iron reduction played a vital role in the cell's metabolism or was a by-product of conditions created in the medium by the growth of the culture.

The effect of anaerobiosis was tested by inoculating the isolates into two sets of tubes of B10 broth. One set was sealed with a 2 cm. depth of 2% water-agar. Speed of reduction was observed visually to determine whether an increased degree of anaerobiosis caused faster and more extensive reduction.

Isolate 92 was inoculated into a one quarter inch depth of B7 broth in a 250 ml. Erlenmeyer flask with a cotton plug. When reduction occurred the flask was shaken at rapid speed on a Northcott rotary shaker to determine the effect of maximum aeration on the reduction process.

The effect of change in pH was determined by inoculating isolates into B10 broth at pH 7.0 and estimating the resulting pH after 4 days of incubation by a Brom-Thymol Blue spot test.

The following experiment measured the effect of anaerobiosis, pH change, and drop in oxidation-reduction potential on the reduction of Fe^{+++} . The apparatus designed for this experiment is shown in Figure 1.

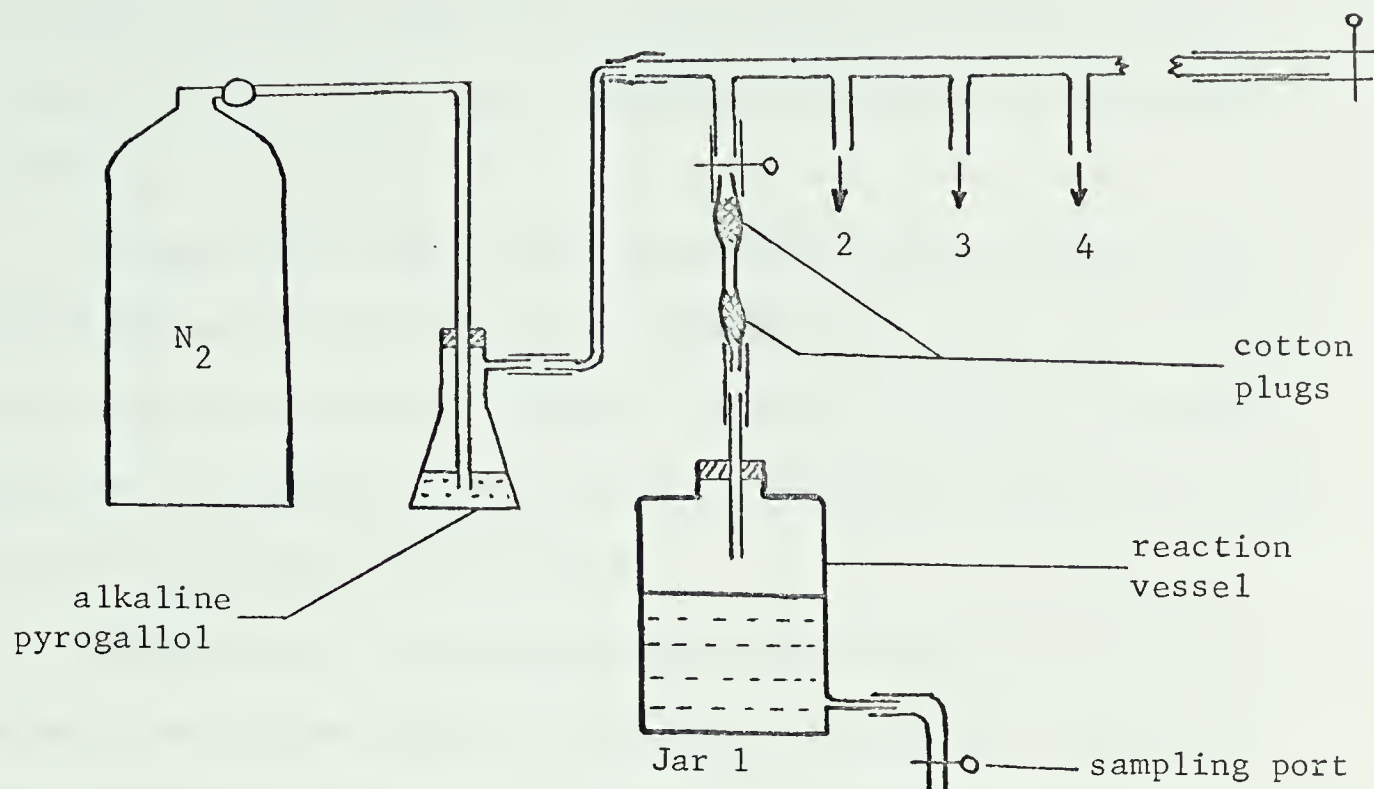


Figure 1. Apparatus for quantitative experiments with iron reducers under anaerobic conditions.

200 ml of B10 broth were added to Jars 1 and 2 and the reaction vessels steam-sterilized. 10g of pyrogallol in 100 ml. 0.1 N NaOH was utilized to eliminate residual oxygen in the commercial grade nitrogen. Jar 2 was inoculated with a 12 hr. culture of isolate 92 and to both jars, to satisfy CO_2 requirements for growth, 5 ml. of a sterile 1% $CaCO_3$ suspension was added. Jar 1 was left uninoculated and to maintain sterility 2 drops of toluene were added. Both jars were thoroughly flushed with the oxygen-free nitrogen before stoppers were inserted. It was found that the system could be maintained under a positive pressure of about $\frac{1}{2}$ lb. of N_2 . When the system had

stabilized at this level, Jar 1 was sealed off from Jar 2 with a clamp to prevent the toluene vapors from entering the inoculated culture.

Samples were taken into clean beakers from a sampling port at the bottom of each jar. When sampling Jar 1, Jar 2 was clamped off and the clamp of Jar 1 opened. It was found that the positive pressure of nitrogen allowed samples to be removed easily, with the sample being replaced by nitrogen.

Sampling was done periodically over a period of 30 hours. A separate sample was taken for each separate analysis. Total iron was determined in the first sample by the method of Krishna Murti et al (1966). Fe^{++} was measured by the method outlined above. pH was determined on a Beckman Zeromatic pH meter with glass and calomel electrodes. Redox potentials were measured on a Copenhagen Radiometer with platinum and calomel electrodes. It should be noted that the redox potential measurements were fairly crude since 1) the standard was distilled water (which was known to have a redox potential of 232 mv.) and 2) these measurements should actually be performed under anaerobiosis. Nevertheless, general trends could be observed with this procedure.

To determine whether metabolic products or extracellular enzymes of these organisms reduce iron, culture fluids of the following representative isolates were collected: 92, which shows poor growth in control medium A3 under anaerobic conditions; 130, which shows fair growth anaerobically in A3; 14-4, with good growth in A3; and

B. mycoides, which is a poor reducer in B10. (The significance of this grouping of organisms will be explained later.)

These isolates were grown in large test tubes containing 20 ml of B10 and in another series containing the control medium A3, which contains only micronutrient quantities of iron. Both aerobic and anaerobic growth were permitted in these tubes. After 4 days, these cultures were centrifuged in the cold (5°C to 10°C) at 10,000 r.p.m. (12,000X g) for 10 minutes in an SS34 head on a Sorvall RC2 centrifuge. 5 ml. of each "cell-free" culture fluid were boiled for 10 minutes to destroy enzymatic activity and allowed to cool. Controls were set up in the same manner to include centrifuged boiled and centrifuged unboiled B10 and A3. Fe^{++} determinations were then performed on a bottle of sterile B10 and the boiled and unboiled B10 supernatants. 2.5 ml. of each of the 20 supernatants were mixed with equal volumes of sterile B10 and allowed to react for 2 hours, after which determinations of Fe^{++} were performed.

Iron Reduction and Cellular Metabolism

In order to determine whether iron played a role in the metabolism of the iron reducing cell, particularly as a terminal electron acceptor under anaerobic conditions, the isolates were inoculated into tubes of B10 and control A3 media. Several runs were made of this experiment. Some runs utilized a Labline or a National Appliance anaerobic chamber and the iron wool method of anaerobiosis (Parker, 1955) with 2 to 5 day incubations of the

isolates. Growth was estimated visually. Other runs involved sealing the tubes with a 2 cm. depth of 2% water-agar, and provided that there was no apparent leakage of air or slippage of agar caps, were just as successful. The isolates were also grown in unsealed tubes of A3 to ensure that this medium was adequate for aerobic growth.

Isolate 14-4, which upon visual inspection grew as well anaerobically in A3 as in B10, was grown for three days in agar sealed A3 and B10 to determine whether anaerobic growth was benefitted in the presence of Fe^{+++} . A viable count of this isolate in each medium was performed in triplicate utilizing serial ten-fold dilutions in sterile water blanks and plating of 0.1 ml aliquots on plate count agar. Counts of colonies were made after three days incubation.

Isolates 92, 130, 14-4, and B. mycoides, representing different classes of iron reducers according to extent of reduction in B10 and anaerobic growth in A3, were grown in unsealed tubes of B10 for 4 days. Fe^{++} was determined in order to discover quantitatively the difference in effectiveness of reduction by these organisms $[\text{Fe}^{++}]$ in a sterile tube of B10 was also determined.

Isolate 92 was inoculated into duplicate tubes containing the following media: Vitamin B12 broth for denitrifiers; Vitamin B12m, which is Vitamin B12 modified by substituting $(\text{NH}_4)_2\text{SO}_4$ for KNO_3 so that it can be used as a control for the denitrifier broth; Butlin's medium for sulphate reducers amended with 3 drops of sterile 10% Na_2SO_3 per tube; and Butlin's medium alone, used as a control for Butlin's + SO_3^- .

All were inoculated with isolate 92. One tube of each medium was sealed with agar and the other left unsealed to ensure that all were adequate as growth media with aerobic conditions. After three days, growth was examined in all tubes, and the denitrifier broth tested for NO_2^- with Trommsdorf and diphenylamine spot tests.

The relationship of growth and reduction of iron was investigated for isolate 92. 8 ml. screw-capped vials containing B7 (ferric ammonium citrate) broth were inoculated with isolate 92, fully filled with sterile B7, sealed, and allowed to incubate at room temperature. A standard curve for Fe^{++} was run in control A1 medium, and total iron in B7 determined by the method of Krishna Murti et al (1966). At periodic intervals one vial was opened, Fe^{++} determined by the method described above, and viable population determined by triplicate plating of aliquots of serial dilutions of the culture on plate count agar. A plate count was required since the dark and changing color of B7 and the formation of precipitates made measurements of optical density impossible.

The next growth curve was run in B10 medium, and in this case growth in B10 was compared against growth in control A3 in order to show that not only are growth and reduction related, but that growth is dependent on the presence of Fe^{+++} . The same procedure was used as with the run in B7, except that 8 ml. screw-cap test tubes were used and the standard curve run in A3. A sterile control with B10 was utilized to measure reduction of Fe^{+++} without inoculum. It should be noted that the first three results of $[\text{Fe}^{++}]$ in B10 at

the beginning of the reduction curve were read at 10, 8, and 6 hours respectively after addition of the o-phenanthroline reagent. This experiment was carried out before it was realized that autoredution of the sample occurred after 4 hours in the buffered color-developing solution. These early results, therefore, were adjusted approximately to the B10 autoredution curves of Figure 2.

Resting cell studies:

Qualitative resting cell studies were carried out to determine whether cells in a relatively steady state of maintenance metabolism could reduce iron. The base for the media used was the B19 used for the carbohydrate utilization study with $(\text{NH}_4)_2\text{SO}_4$ deleted to prevent growth.

Isolates for which a simple carbohydrate energy source had been found were grown for 2 days on B10 agar flats. Cells were harvested in sterile saline (0.85% NaCl), centrifuged at 10,000 r.p.m. (12,000 X g) at 5°C. to 10°C. for 10 minutes, washed in 20 ml. sterile saline, and recentrifuged. Precipitates were suspended in B19 minus nitrogen and starved for two hours to consume intracellular storage products and surplus nitrogen. The cultures were recentrifuged, resuspended in saline, and the optical densities of the suspensions read at 600 mu on a Bausch and Lomb Spectronic 20.

To each of 22 ml. screw-cap tubes containing B19 resting cell base medium 1 ml. of sterile 10% dextrose, Na-lactate, or sucrose was added. Six 8 ml. screw-cap tubes with B19 r.c. base were each amended with 0.5 ml. of sterile 5% Na-pyruvate. 1 ml. of the resting

cell suspensions were inoculated in duplicate into their appropriate media (Table 4) to yield final cell optical densities of 0.1 to 0.4. Duplicate controls were left for each medium. A duplicate of each paired set was then boiled for 10 minutes to destroy enzymatic activity. All tubes were completely filled with sterile B19 r.c. base, sealed, and observed after 24 hours.

Attempts were made to discover whether a stoichiometric relationship exists between the quantities of lactate utilized and iron reduced. For all experiments the apparatus of Figure 1 was utilized. In a trial run, isolate 92 was grown for 2 days on B10 agar in Roux bottles and medical flats and resting cells prepared in the manner described above.

The constituents and procedures for the 5 jars set up for this experiment were as follows:

Jar 1: 30 ml. B19 resting cell base medium + 55 ml.
distilled water. Sterilize. Add 5 ml. sterile
4000 ppm. Na-lactate + 10 ml. resting cells in
saline. $\text{Fe}^{+++} = 3.6 \text{ mM/l.}$, lactate = 2.2 mM/l. ,
cell O.D. = 0.4.

Jar 2: As in Jar 1, but cell O.D. = 0.2.

Jar 3: As in Jar 1, but substitute A19 resting cell base
medium for B19 r.c. base as control (A19 r.c. base
substitutes K_2HPO_4 and a micronutrient quantity of
iron for FePO_4 .) Cell O.D. = 0.4.

Jar 4: As in Jar 3, but cell O.D. = 0.2.

Jar 5: As in Jar 1, but boil cells for 10 minutes before adding to jar.

Samples were taken at 0, 2, and 4 hours after inoculation, with the jar shaken well before sampling. Fe^{++} was determined by the method outlined above and the standard curve for Fe^{++} run in A19 r.c. base. Samples taken for the lactate determination were centrifuged at 10,000 r.p.m. in the cold to remove cells. 2 ml. of the supernatant was diluted to a 50 ml. volume, and 1 ml. aliquots taken for lactate determination by the method of Neish (1952). (This method involves the oxidation of lactate to acetaldehyde with concentrated H_2SO_4 , and color development with p-OH-diphenyl.) A standard curve for lactate, using dry Li-lactate, was run from 0 to 10 ppm. in a 25 fold dilution of B19 r.c. base and color read at 570 m μ . on a Bausch and Lomb Spectronic 20.

Since difficulties were encountered with the lactate determination, the effect of the temperature of the sample before addition of H_2SO_4 was investigated. Standard curves using Li-lactate were run in distilled water. One set of standards was cooled to 5°C., another left at room temperature, the third heated to 50°C., and the fourth heated to 65°C. before addition of H_2SO_4 . Careful attention was given to elimination of as much variability as possible in the syringing technique.

The second resting cell experiment used a modified B19 resting cell base medium (B20). In B20 the pH was raised to 7.8 after sterilization in order to eliminate the high background of Fe^{++}

present in B19. The FePO_4 content was adjusted (7×10^{-3} M/l.) so that the full extent of the standard curve could be utilized. Unlike the first run, the medium was not diluted with water since it was thought lysis of cells in the diluted buffer had contributed "background" material, thereby causing a false increase in the lactate measurement. Finally, to delay the precipitation of $\text{Fe}_3(\text{PO}_4)_2$, 1% Na-citrate was added as an organic material which would stabilize the iron solution and, as demonstrated earlier, not be utilized as an energy source by isolate 92. The control medium was A20, which again substituted K_2HPO_4 and a trace of FeSO_4 for the FePO_4 of B20.

4 days cells of 92 were harvested in sterile saline from B10 agar flats, and resting cells prepared as before. Sterile constituents were added to the jars of Figure 1 as follows:

Jar 1: Control for Fe^{+++} reduction, no lactate. 180 ml.

B20 base + 5 ml. cells + 15 ml. distilled water.

Jar 2: As in (1) but 10 ml. of 1% lactate. $\text{Fe} = 7.2 \text{ mM/l.}$,

lactate = 3.0 mM/l. , cell O.D. = 0.08.

Jar 3: Control for endogenous metabolism of lactate. As in

(2) but substitute A20 for B20.

Jars 4, 5, 6: As for (1), (2), and (3) respectively but halve cell O.D. to 0.04.

Standard curves for Fe^{++} were run in A20, and two sets of standard curves were run for lactate — in B20 and in control A20 in case iron had an effect. Samples of Fe^{++} were determined as before. 5 ml. lactate samples were centrifuged at 10,000 r.p.m. in

the cold and 2 ml. of supernatant withdrawn to be diluted for determination. Lactate determinations were done in triplicate and the H_2SO_4 added to samples at room temperature.

Since lactate results were still too high, the cells were regrown for 2 days on the old B10 agar flats flooded with B10 medium and resting cells prepared as before. Jars 1, 2, and 3 were set up as in the last run, and Jar 4 was set up with all of the constituents of Jar 3 (the control for endogenous metabolism of lactate) except for the omission of lactate. Cell O.D. in all four jars was 0.045, a deliberately low quantity of cells to slow the reaction and decrease cell "background" material in the lactate determination.

In this final run, standard curves were run in the B20 minus lactate (Jar 1) and A20 minus lactate (Jar 4) each time a determination of lactate was made, thus running the standard curves with the cells present. This had been avoided as long as possible, since this meant that the effective range of the standard curve for lactate was now from 0 to 5 ppm., halving the effective range of the determination and doubling the error. Also, since "background" was still high, the samples were diluted 100-fold which decreased the effective range of determination even further. Standard curves are shown in Appendix III.

Inhibitor studies:

Studies with CN^- , N_3^- , and CO were undertaken to determine whether Fe^{+++} was reduced by a cytochrome system or by an anaerobic dehydrogenase reaction. Test tubes containing 4.5 ml. of B10 broth

were amended with 0.5 ml. sterile KCN or NaN_3 solutions and thoroughly mixed to yield media with the following concentrations of inhibitor: no inhibitor (control); 10^{-4} M, 10^{-3} M, and 10^{-2} M KCN; and 10^{-4} M, 10^{-3} M, and 10^{-2} M NaN_3 . These very high concentrations of inhibitors were required because of the masking effect that high quantities of iron have on the action of these inhibitors (Hochster and Quastel, 1965). Each isolate was inoculated into a set of these series and the tube sealed with 2% agar. Reduction was examined daily by visual inspection.

Since reduction of iron occurred in 10^{-3} M KCN, it was desirable to determine the effect of this concentration of cyanide on aerobic metabolism in B10. 125 ml. flasks containing 10 ml. of B10 with 10^{-3} M KCN were inoculated with all isolates, incubated aerobically on a rotary shaker, and growth examined after 2 days.

Isolates were inoculated into unsealed tubes of B10 broth and placed in a Labline anaerobic chamber. The chamber was flushed three times with nitrogen, evacuated, and filled with pure CO gas. To ensure sealing a negative pressure of $1\frac{1}{2}$ lb. was left in the chamber. The iron wool method of anaerobiosis (Parker, 1955) was used to ensure provision of CO_2 and eliminated residual O_2 . Isolates were inoculated also into B10 tubes which were left outside the chamber. These were all reduced by the end of three days, and one extra day was allowed before examining the tubes in the chamber. The extent of reduction was estimated by visual inspection and a spot test with 2% α - α' -dipyridal.

Adaptation of Isolate 92 From Aerobic Conditions to the Anaerobic Fe^{+++} -reducing Process

In order to determine the period required for isolate 92 to adapt from aerobic conditions to the anaerobic Fe^{+++} -reducing process, this organism was inoculated into a tube of A3 control medium and left for 10 days (Nearly all of the growth by 92 in this medium is aerobic.) The culture was transferred to 3 ml. of A3 and incubated aerobically on a Burrell wrist-action shaker. During the next 10 days the culture was transferred to fresh A3 and grown on the shaker 5 times in order to provide a culture which had not been in contact with anaerobiosis or more than micronutrient quantities of Fe^{+++} for many generations. The last transfer was made 16 hours before the beginning of the adaptation experiment. Parallel to this series, 92 was run through several transfers in B10, the last transfer being made to B10 and agar-sealed 16 hours before the following experiment.

The anaerobic apparatus of Figure 1 was set up with 4 sterile jars containing 100 ml. of B10 and, to provide CO_2 , 5 ml. of a sterile 1% suspension of CaCO_3 . 2 jars were inoculated with the 16 hour B10 culture and 2 jars with the 16 hour A3 culture. The jars were flushed with N_2 , sealed, and allowed to stabilize at a positive pressure of $\frac{1}{2}$ lb. Fe^{++} determinations were performed by the o-phenanthroline method described earlier.

Ecology and Function of the Iron Reducing Population of Soil

The Effect of Various Environmental Factors on the Population of Iron Reducers in Soil

Technique for estimating the iron reducing population:

The Most Probable Number technique was used to estimate the numbers of iron reducing organisms in soil. 10 g. samples of 4 surface soils were shaken vigorously for 2 minutes by hand in 90 ml. sterile water blanks. No time was allowed for the precipitates to settle and serial tenfold dilutions were made up to 10^{-6} dilutions. 5 tubes of B7 medium were inoculated for each dilution, 1 ml. per tube. The tubes were left unsealed and examined daily for visible reduction in order to determine the time required for the maximum number of tubes to reduce.

Since B10 was utilized for later MPN determinations, it was important to determine whether results in the B7 (ferric ammonium citrate) medium were the same as for B10 (ferric phosphate). A black chernozemic Ah sample was diluted in the manner described above and three replicates of 5 unsealed tubes per dilution were inoculated for each medium tested. After one week's incubation at room temperature, tubes showing visible reduction were counted and numbers of iron reducers calculated from a table of Most Probable Numbers.

Studies of field samples:

A general study was made of the MPN of iron reducers in various

soil samples collected over a period of two years. (Description of samples and media used are given in Table 15). Samples were collected in sterile jars which were tightly closed to prevent moisture loss. The samples were stored at 5°C. or lower as soon as they were brought in from the field and MPN determinations done as soon as possible. Moisture content was determined by drying a weighed portion of the soil at 105°C. to 110°C. overnight in an oven. For the Minimal Podzol sample (Table 16) pH was determined by the soil paste method and organic matter content by heating the samples to 400°C. in a muffle furnace for 4 hours.

After this survey had indicated the general results which could be expected from field samples, a more detailed investigation of the habitat of iron reducing populations was made. Samples of an Orthic Black Chernozem, a Black Solod, an Eluviated Black, a Dark Grey Wooded, and a Grey Wooded soil were collected from the Edmonton region in September of 1967. Triplicate determinations of the iron reducing population were performed by the MPN method. Moisture content of the samples was determined after drying weighed portions in a 105°C. oven overnight. The samples were air-dried, ground to pass a 2 mm. sieve, and the following analyses performed. Total N and total cation exchange capacity were performed by Kjeldahl methods, pH by the soil paste method, total C on a Leco furnace, and free iron by the oxalate extraction method. (All of these methods are outlined in Soil Laboratory Analysis, 1967.) Inorganic C was determined on the Smolik calcimeter (Bascombe, 1961) on samples with a pH greater

than 7.0. Soluble iron was determined by the method of Olson and Carlson (Olson, 1965), which involves an o-phenanthroline colorimetric determination on a 1 N, pH 4.8 NH_4^+ -acetate extract of the soil sample.

Laboratory studies:

Several ecological experiments were carried out in the laboratory to determine the effect of various environmental conditions on the population of iron reducers in soil. The soil utilized for these experiments was a black Chernozemic Ah soil (sample G). pH, moisture content, total C, total N, cation exchange capacity and free iron content were determined as described above for field samples.

The range of temperature over which microbial iron reduction could occur was investigated. Tubes of B10 broth were inoculated with pure isolates 92 and 130, a mixed culture JB, or 0.1 g. of a B horizon of a Humic Gleysol (sample IR16) or a heavily manured garden soil (sample IR2, from Vulcan, Alberta). A series of each culture was incubated with their controls at the following temperatures: 2°C. (cold room), 5°C. (cold room), 10°C. (refrigerator), room temperature (22°C. to 26°C.), 37°C. (oven), 50°C. (oven), and 65°C. (water bath). The length of time required for strong visible reduction to occur was observed. After reduction of IR2 and IR16 cultures, the experiment was repeated with transfers from these soil cultures. Several transfers were made of the 65°C. IR2 culture. When this thermophilic iron reducing culture was ultimately lost, attempts to isolate one were

made using samples of IR2 and G soils in B10 at 65°C. in a waterbath.

6 plastic bottles, each with 100 g. of air-dry sample G soil plus 100 ml. of distilled water, were tightly sealed and incubated for 4 weeks at the following temperatures: 5°C. and 15°C. (refrigerators), room temperature, 40°C. and 50°C. (ovens), and 65°C. (waterbath). After 4 weeks samples were thoroughly mixed. Total "soluble" iron was performed by the method of Olson and Carlson (Olson, 1965), using blanks to correct for the color of the soil filtrate. Fe^{++} was determined by the same method, except that hydroxylamine hydrochloride was omitted. A standard curve for iron was run in the NH_4^+ -acetate extracting solution (Appendix II). A count of iron reducers was performed by MPN technique on 10 g. of the sample using B10. After inoculating B10 tubes for the counts, the tubes were incubated as follows: those from the 5°C. samples were incubated at 5°C. for 3 weeks, the 15°C. at that temperature for 2 weeks, room temperature for 1 week, 40°C. for 1 week but with a daily check to prevent re-oxidized tubes from being missed, and 50°C. and 65°C. for 5 days with daily checking. At the same time isolate 92 was inoculated into B10 tubes and incubated at these temperatures.

An experiment investigating the effect of soil reaction on the iron reducing population was begun by weighing 10 g. of air-dried soil G into 50 ml. beakers. 20 ml. of various strengths of HCl or NaOH were added to the soil and mixed well. pH was measured in each well-mixed sample with a Beckman Zeromatic pH-meter at $\frac{1}{2}$ hr. and 1 day to

determine the initial conditions with which the organisms had to contend. (These measurements are given in Table 19.) HCl and NaOH were used in preference to H_2SO_4 and KOH since these last two reagents would add nutrients to the soil. The beakers were covered with parafilm and incubated at room temperature. After 5 weeks, pH was measured and total soluble iron, and soluble Fe^{++} were determined by the methods outlined above.

The MPN of iron reducers was determined as follows. 60 ml. volumes of B10 broth were titrated with 0.1 N NaOH or 0.1 N HCl to the final reactions of the samples, and the required number of sterile tubes of B10 amended aseptically with the appropriate volumes of sterile 0.1 N NaOH or 0.1 N HCl to the desired reactions. Standard MPN technique was then followed. 2 tubes at each pH were left as controls, another 2 were inoculated with isolate 92 to determine the range of pH within which it could reduce iron.

After MPN was determined, the pH of several selected reduced cultures were measured with a Beckman combination glass and calomel electrode.

To examine the populations in these cultures, 100 ml. portions of B10 broth were amended to the final reactions of the incubated soil samples. 2 g. of agar was added to each, and the broths autoclaved. The pH was taken of aliquots of these which had no agar added but were autoclaved. There was change in pH (Table 20) but the resulting reactions were acceptable, since they were a compromise between the final reactions of the soil samples and the final reactions

of the selected B10 cultures from these samples. B10 plates at these various reactions were poured, and the selected cultures plated for single colonies. The agar did not set at pH 4.0, so semisolid tubes were set up as growth media for cultures from this reaction. Also inoculated with all of these cultures were B10 plates at neutral pH.

To measure the effect of adding free iron to the soil, three jars containing 50 g. of sample G were prepared. 2.0 g. Fe_2O_3 was added to one jar, 4.0 g. Fe_2O_3 to the second, and none to the third. All jars were raised to 100% moisture, sealed with parafilm and incubated for five weeks at room temperature.

The effect of organic matter addition was investigated by preparing three jars containing 100 g. of soil G to the first jar, 2 g. of dry ground oat straw was added; to the second, 2 g. of dry ground alfalfa; and the third was left as control. The jars were brought to 100% moisture, sealed with parafilm, and incubated at room temperature for five weeks.

The effect of increased moisture content of the soil (and, therefore, anaerobiosis) was investigated by weighing air dry soil G into jars and adjusting moisture content to 2.2% (air-dry), 10%, 20%, 50%, 100%, 200%, and 400%. The jars were covered with parafilm and an airhole punched in the centre of each cover. The jars were incubated at room temperature for five weeks and brought up to the required weight with distilled water every week.

Determination of MPN of iron reducers, total soluble iron, and soluble Fe^{++} were performed for these last experiments as before,

Samples from the more aqueous soils in the moisture experiments were removed with a pipette after thorough mixing.

Solubilization and Reduction of Iron Compounds by Plant Extracts and Iron Reducing Cultures

Bloomfield (1954b) showed that aspen poplar (Populus tremuloides) leaf constituents could reduce iron. To compare the nature of reduction by plant extracts and isolate 92, 7.5 g. of summer-picked air-dried aspen poplar leaves were steam-sterilized in 75 ml. of water to form a 10^{-1} dilution of leaves. The supernatant was carried through serial ten-fold dilution up to 10^{-6} dilution. 5 tubes of B10 broth (each containing 6 ml.) were amended with 1 ml. apiece of the 10^{-1} dilution of the leaf extract. The same procedure was followed for the other dilutions. 2 tubes of each set of 5 were also inoculated with isolate 92. After 2 days of incubation all tubes were tested for Fe^{++} by a spot test with 2% o-phenanthroline.

It was desirable for the following solubilization experiments to determine whether the 3% AlCl_3 extraction method (Ignatieff, 1941) or the 1 N, pH 4.8, NH_4^+ -acetate extraction method (Olson, 1965) was superior in liberating iron from a plant-extract complex. 2 g. of dried, ground, fall-picked aspen poplar leaves were heated in 20 ml. of water to near boiling point for 45 minutes. 1 ml. of the leaf extract was added to 6 ml. of B10 broth and mixed. Two 1 ml. aliquots of this mixture were shaken with 4 ml. of 1 N NH_4^+ -acetate (pH 4.8) for 30 minutes on a Burrell wrist shaker. A further two 1 ml. aliquots were shaken with 2.5 ml. 3% AlCl_3 . The remaining two

aliquots were not treated. Standard curves were prepared for Fe^{++} in the appropriate dilutions of A3, A3 + AlCl_3 , and A3 + NH_4^+ -acetate (Appendix III), and Fe^{++} and total iron determined by the o-phenanthroline methods described earlier in untreated B10 and in the B10-leaf extract samples.

That iron reducing cultures can reduce soluble iron has been established. The following experiments were designed to determine whether these organisms could solubilize Fe_2O_3 and other insoluble compounds in order to obtain Fe^{+++} as a terminal electron acceptor, and whether they were more effective than plant materials in solubilizing and reducing Fe^{+++} . 26 of 30 eight ml. screw-cap vials containing 0.1 g. of powdered Fe_2O_3 and full of A3 control medium were inoculated with the stock cultures. The 27th was left as an uninoculated control and kept sterile with a drop of toluene. Vials 28 and 29 were amended with 0.1 g. of freshly picked, chopped bromegrass (Bromus inermis). (Bloomfield in 1951 found that grass extracts could solubilize and reduce Fe^{+++} .) Vial 28 was kept sterile with a drop of toluene to determine the effect of grass alone compared to microbial action. Vial 29 was inoculated with 0.1 g. of lawn soil to discover whether the soil microflora, while decomposing grass, were more effective than the pure cultures in solubilizing iron. Vial 30 measured reduction of Fe_2O_3 by 0.1 g. of soil. Vials 31 and 32 were controls for the soil inocula, containing A3 broth and 0.1 g. of soil, but no Fe_2O_3 . Vial 31 was autoclaved; vial 32 was left non-sterile.

Isolates 92, 130, and 14-4 (representing poor, fair, and good

growth, respectively, by isolates in A3 under anaerobiasis) were inoculated into three of four 22 ml. screw-cap tubes containing A3 and 0.1 g. of crushed hematitic rock (from Waterton National Park). The fourth tube was a sterile control. The purpose here was to determine whether those organisms which grew more heavily in A3 under anaerobiasis than isolate 92 could solubilize more Fe^{+++} .

Also tested with these cultures were iron ores from the Clear Hills area. A series of these samples contain 27.9% total iron and 13% to 36% organic matter. The iron is present as hydrated ferric and ferrous oxides and as siderite (FeCO_3). The sample CH₅ that was used in this study contained more ferrous iron than did the CH₁₂ sample used (Baadsgaard, 1967). Experiments with ore samples CH₅ and CH₁₂ were set up with the same inocula and in the same manner as with the hematitic rock, with 0.1 g. of ore per 22 ml. tube.

The 32 tubes in the Fe_2O_3 experiment, and the 12 in the hematite and iron ore experiments were completely filled with sterile A3, sealed, and incubated at room temperature. After 10 days, the entire contents of each tube were shaken rapidly for 30 minutes in an equal volume of 1 N, pH 4.8, NH_4^+ -acetate on a New Brunswick rotary shaker. Suction filtration of the extract followed, through Whatman No. 2 filter paper until filtrates were clear. Determinations of Fe^{++} and total soluble iron were conducted by the o-phenanthroline methods discussed earlier.

The Role of Iron Reducing Bacteria in Gleization

Experiments were conducted to compare extent of gleization

obtained with sterile conditions, plant materials, and pure and mixed cultures. Four 22 ml. screw-cap test tubes, containing 1.0 g. of a black Orthic Chernozem Bm soil sample (ground to 2 mm.) and A3 medium, were steam-sterilized. The Bm horizon sample, obtained from Dudas (1968), contained 0.03% oxalate extractable free iron, 0.09% N, and 1.00% C. One tube was kept sterile with a drop of toluene, two others were inoculated, respectively, with isolate 92 and isolate 14-4, and the fourth was inoculated with 0.1 g. of lawn soil.

Four 22 ml. screw-cap tubes containing 1.0 g. of the Bm sample and distilled water were prepared. To one tube, a few drops of toluene were added to create sterile conditions. The second tube was allowed to incubate with the organisms already present in the Bm soil. The last two were amended with 0.1 g. of dried chopped brome grass, of which one was treated with a few drops of toluene. After 10 days incubation at room temperature, total soluble iron and soluble Fe^{++} were determined as in the solubilization experiments.

To show visible comparison of gleying under different conditions, 5.0 g. of the Bm soil was weighed into each of twenty-eight 22 ml. screw-cap tubes. 5 series were set up with the following amendments:

- 1: distilled water; 6 tubes.
- 2: distilled water + 1.0 g. of dry, ground, fallen aspen poplar leaves; 6 tubes.
- 3: distilled water + 0.1 g. aspen poplar leaves; 6 tubes.
- 4: A3 medium; 6 tubes.
- 5: A3 medium; 4 tubes.

Two tubes of each series were autoclaved at 15 lb. pressure for 20 min. on 2 successive days. 2 other tubes of each series were sterilized by γ -radiation at 3/4 megarads/hr. for 8 hr. on 2 successive days in a Cobalt Gammacell 220 (Atomic Energy of Canada). This was done to ensure that with these fairly large quantities of soil complete sterility would be obtained in at least one control per series, and to ensure that with the changes in the soil effected with these drastic sterilization methods, results from alternatives could be examined. All tubes were completely filled and sealed. The tubes of series 5 were inoculated with isolate 92. After incubation and observation for three months, selected tubes were photographed by University of Alberta Photoservices.

Investigation of Siderite Formation by Iron Reducing Cultures

In conjunction with Dr. P. Fritz (post-doctoral fellow of Department of Geology), who was interested in microbial formation of siderite (FeCO_3), further studies were made of iron reducing culture precipitates. Cultures of isolate 92 and lawn soil were incubated in unsealed and sealed tubes of B10 broth for one week. Isolate 92 was grown also in an agar-sealed tube of B19-lactate (ferric phosphate and sodium lactate) medium. Resulting precipitates were microscopically examined.

When the high phosphate concentrations in B10 and B19 resulted in ferrous phosphate precipitates only, B7 (ferric ammonium citrate) medium was utilized in place of B10. Isolate 92 and lawn soil were

inoculated into two sets of unsealed tubes and screw-capped vials of B7. The precipitates of one set of each culture was examined after 4 days of incubation, and the other set after two weeks by X-ray analysis.

Iron Reduction and Iron Corrosion

Sulphate reduction is known to cause iron corrosion (Starkey, 1945), and corrosion caused by nitrate reduction has been observed in our laboratory. A series of experiments were undertaken to determine if iron reduction could cause an analogous result.

To determine whether some of the nitrate reducing, iron corroding organisms from Dr. F. D. Cook's collection could reduce Fe^{+++} , a few selected corrodors were inoculated into B10 broth and their rate of reduction compared with isolate 92.

To determine whether iron reducers could corrode iron, several nitrate reducing and non-nitrate reducing iron reducers were inoculated into iron corrosion medium (essentially iron wire, KNO_3 , plus tryptone) and their action compared to isolate '1', the most effective of Cook's corrodors. The wire for the iron corrosion medium was dried for 2 hr. at $50^\circ\text{C}.$, weighed, and sterilized in 30 ml. vials. The remainder of the medium was added aseptically, the organisms inoculated, and the vials completely filled with medium and sealed. As well as 2 uninoculated controls, 2 sterile controls were set up with 0.1% KNO_2 . After one week of incubation at room temperature, the wires were removed, cleaned of adhering crusts, dried at $50^\circ\text{C}.$ for 2 hrs., and weighed.

The cultures were tested for NO_3^- and NO_2^- by spot tests with diphenylamine and Trommsdorf reagents.

Finally, those iron corroders which had demonstrated ability to reduce Fe^{+++} and selected nitrate and non-nitrate reducing iron reducers were run through the preceding experiment, but in B10 plus iron wire rather than iron corrosion medium to determine whether the iron reduction process also caused iron corrosion. As well as 2 uninoculated controls, two sterile controls were set up with A3 in place of B10 and containing 1% FeSO_4 .

Further Isolations of Iron Reducing Organisms and Miscellaneous Experiments

Fungi

The collection of fungi obtained from peat soils by Gardner (1967), comprising 55 cultures of Penicillium, Chrysosporium, Cladosporium, Mucor, yeasts, unidentified, and mixed cultures, was inoculated into B7 broth and examined for reduction after two weeks of incubation with spot tests with 2% α - α' -dipyridal. Subsequent transfers of all cultures were made in B7 and B10 broths, and observed for reduction. A few fungi were selected, transferred to B10 broth, incubated for 10 days at room temperature, and analyzed for Fe^{++} by the o-phenanthroline method.

Relationship of Citrate Fermentation and Iron Reduction

None of the stock isolates of iron reducers produce gas in

agar-sealed B10 broth. All were grown in agar-sealed B7 (ferric ammonium citrate) broth and observed for gas production to determine whether any might be citrate fermenters.

Attempts were made to isolate a citrate fermenting iron reducer from lawn soil by enrichment isolation technique in B7 broth.

Actinomycetes

Several actinomycetes obtained on B7 and B10 plates during isolation were inoculated into B10 broth and observed for reduction.

Attempts to Isolate 92 and 1-8 from Soil

Isolates 92 and 1-8 have been found only once on B7 and B10 plates—at the time of their original isolations. Several attempts were made to isolate these organisms by utilizing their unique properties among the iron reducing isolates. Lawn soil dilutions were inoculated into B19-lactate broth tubes and agar-sealed. These soil dilutions were also inoculated into Butlin's medium for sulphate reducers plus Na_2SO_3 . After one week all of these primary cultures were plated for single colony on B10, and also transferred to B19-lactate broth and agar-sealed. After 1 week the enriched cultures were plated on B10.

V. RESULTS AND DISCUSSION

Identification and Characterization of Iron Reducing Isolates

Effective Iron Reduction in Iron Medium

All of the selected isolates, with the exception of isolate 3 and the taxonomically known Bacillus cultures, were considered to be effective iron reducers. An iron reducer was judged to be effective if changes in the medium indicative of strong reduction (as described in Chapter III) were visible, and if there was a deep red color resulting from a spot test of a drop of the culture with 2% orthophenanthroline or 2% α - α' -dipyridal. An example of the iron reduction shown by isolate 92 thirty hours after inoculation is shown in Plate 1. Isolate 3 and the named Bacillus cultures often show a certain amount of reduction, but this reduction neither appears as consistently nor develops as strongly as with the effective isolates.

Identification of Type Isolates

Since all of the cultures were capable of both anaerobic and aerobic growth, they are facultative aerobes. All isolates are rod-shaped, and several have demonstrated motility (Table 2).

Ability to form spores was demonstrated by most isolates but required a great deal of observation due to the inconsistent occurrence of sporulation and, when spores were seen, the very sparse numbers of spores produced in broths and on plate. Better



Plate 1. Reduction of ferric iron in B10 medium by isolate 92.
Magnification = 2X

Left: uninoculated control

Right: 30 hour culture of isolate 92 incubated at room temperature

Table 2. Cell morphology, motility, and spore formation by iron reducing isolates.

Isolate	Motility	Morphology and size (μ) of vegetative cells	Spores		
			Growth after pasteurization in the following broths		
			B10	A3	Vitamin B12
1-3	—	rods: 0.5-0.7 X 2.5-5	+	+	—
1-4	—	rods: 0.5-0.9 X 2.5-9	+	—	—
1-6-2	—	rods: 0.5-1 X 2-9	+	—	—
1-8	+	small rods: 0.2-0.5 X 1-3.6	—	—	—
1-10	—	rods: 0.4-0.7 X 3-5	+	—	—
1-12-2	—	rods: 0.3-0.6 X 3-4.5	+	+	+
3	—	rods: singly and in chains 0.5-0.9 X 3-4.5	+	+	+
6	+	rods: 0.3-0.7 X 2-5	+	+	—
7-2	—	rods: some tend to filamentous 0.3-1.2 X 2-9	—	—	—
8-2	—	rods: often in chains 0.4-1.5 X 2-6	+	+	+
12-4	+	rods: 0.3-0.4 X 2-3.4	+	+	+
14-4	—	rods: 0.4-0.5 X 2-3	+	—	—
19	+	rods: 0.3-0.7 X 1.8-6	—	—	—

Isolate	Spores					Classification
	Seen under microscope	Shape of spore	Size (μ) of spore	Position in cell	Swelling of cell	
1-3	+	oval	0.6-1.2 X 1.2-1.8	central to subterminal	+	<u>Bacillus</u> Group 2
1-4	+	oval	0.4-1 X 0.6-1.3	central	+	<u>Bacillus</u> Group 2
1-6-2	+	oval	0.8-1 X 1.5-1.8	subterminal to terminal	+	<u>Bacillus</u> Group 2
1-8	+	oval				<u>Bacillus</u> (very much like isolate 92, Probably Group 2)
1-10	?	nearly spherical?	1 X 1.2?			<u>Bacillus</u>
1-12-2	+	oval	0.8-1.2 X 1.5-2.4	central to terminal	+	<u>Bacillus</u> Group 2
3	+ spores often in chains	oval	0.6-1 X 1.2-1.8	central to terminal		<u>Bacillus</u> Group 2
6	+	oval	0.6-1.2 X 2	subterminal to terminal	+	<u>Bacillus</u> Group 2
7-2	?		1.2 X 2?			<u>Bacillus?</u>
8-2	+	oval	1 X 1.5	central to terminal	+?	<u>Bacillus</u> (Group 2?)
12-4	+	oval	1-1.2 X 1.8-2.5	central to terminal	+	<u>Bacillus</u> Group 2
14-4	+	oval	0.9-1.2 X 1.5-1.8	terminal	+	<u>Bacillus</u> Group 2
19	?		.5 X 1?			<u>Bacillus?</u>

Table 2. (cont'd.)

Isolate	Motility	Morphology and size (μ) of vegetative cells	Spores		
			Growth after pasteurization in the following broths		
			B10	A3	Vitamin B12
22	+	rods: 0.3-0.7 X 2-4.5	+	-	-
92	+	small rods: 0.3-0.5 X 1.5-4	-	-	-
130	-	rods: 0.2-1 X 1.5-5.5	-	-	-
627	+	rods: 0.5-0.6 X 2-6.6	-	-	-
629-3	+	rods: 0.5-0.6 X 2-6.6	+	+	+
633	-	rods: 0.5-0.7 X 2-6.6	+	-	-
634	-	rods: 0.5-0.8 X 2.5-12	-	-	-
635	?	rods: 0.5-0.6 X 2.5-12	+	-	-
636	?	rods: 0.4-0.7 X 3-12	-	-	-
I2	-	rods: singly or filamentous, single rods 0.3-0.6X3-10	+	-	+
<u>B. circ.</u>	+	long and short chains of rods, individual rods 0.8-1X2-12, often tends to filamentous	+	+	+
<u>B. myc.</u>	-	single and chains of rods, 0.6-1.8X3.6-12	+	+	+
<u>B. subt.</u>	-	single and chains of rods, 0.6-1.5X2-9, often tends to be filamentous	+	+	+

Isolate	Spores					Classification
	Seen under microscope	Shape of spore	Size (μ) of spore	Position in Cell	Swelling of cell	
22	+ thick spore wall with sporangium adhering		0.9 X 1.8		+?	<u>Bacillus</u> (probably Group 2)
92	+ thin spore wall	oval	0.5-0.6 X 1-1.2	central to terminal	+	<u>Bacillus</u> Group 2
130	+ thick spore wall	oval	1 X 1.5	central to terminal	slight to +	<u>Bacillus</u> (Group 2?)
627	+ thick spore wall	oval	0.8-0.9 X 1.2-1.5	terminal	+	<u>Bacillus</u> Group 2
629-3	+ thick spore wall	oval	1 X 1.8-2	terminal to subterminal	+	<u>Bacillus</u> Group 2
633	+ thick spore wall	oval	1-1.2 X 1.8	terminal	+	<u>Bacillus</u> Group 2
634	+ thick spore wall	oval	0.8-0.9 X 1.5	terminal	+	<u>Bacillus</u> Group 2
635	-					<u>Bacillus</u>
636	+ thick spore wall	oval	0.5-0.6 X 0.8-1.5	central	?	<u>Bacillus</u>
I2	+ thick spore wall	oval	1 X 1.2-1.5			<u>Bacillus</u>
<u>B. circ.</u>	+ thick spore wall	oval to cylindrical	0.8-1.2 X 2.1-3	central to subterminal	+	<u>Bacillus</u> Group 2
<u>B. myc.</u>	+ thick spore wall	oval to cylindrical	0.6-1.2 X 1.5-2	central to terminal	-	<u>Bacillus</u> Group 1
<u>B. subt.</u>	+ thick spore wall	cylindrical	0.7-1.2 X 1.8-3	central to terminal	-	<u>Bacillus</u> Group 1

success would probably have been obtained using media designed to encourage sporulation (Charney et al, 1951; Halvorson, 1957).

However, all isolates are considered to be Bacillus. The majority have been categorized as Group 2 Bacillus, with sporangia definitely swollen by oval spores (Smith et al, 1952). Group 2 Bacillus include B. polymyxa and B. circulans, the two species stated by Roberts (1947) and Bromfield (1954ab) to have iron reducing properties.

Earlier work by Cook et al (1966), however, with isolates 92, 130, 1-4, and 8-2 showed that no gas from carbohydrates is formed, which eliminated B. polymyxa as a possible identity for these organisms. 8-2 hydrolyzes starch, does not produce indole or acetyl-methylcarbinol, and cannot grow at 65°C., which identifies it as B. circulans. However, the other three cannot hydrolyze starch, will show acid growth in glucose but not in mannitol (Cook et al, 1966), and would fit in the category of B. pulvifaciens except for the fact that their morphology is not the characteristic spindle shape of this organism (Cook, 1968).

Colonial Morphology of Type Isolates on Iron Medium Plate

A description of three day old colonies of selected iron reducing isolates on B10 plate is provided in Table 3. There is no visible reduction of iron in the solid medium with aerobic incubation and although the organisms develop into somewhat smaller colonies on conventional media such as plate count agar or

Table 3. Morphology of three day old single colonies of iron reducing isolates on B10 plate

Isolate	Colonial Morphology				
	Diameter (mm)	Shape	Elevation	Edge	Surface
1-3	4-5	irregular	usually depressed	entire to slightly erose	smooth to slightly rough
1-4	3-4	roughly circular	usually depressed	undulate, occasionally slightly erose or even slightly filamentous	smooth to slightly rough
1-6-2	3	roughly circular	flat or depressed	undulate to slightly wavy	slightly rough to smooth
	4	roughly circular	flat or depressed	slightly undulate	rough
1-8	2-3	circular	depressed	entire	smooth
1-10	1	roughly circular	convex	entire to undulate	smooth glistening
1-12-2	2-4	irregular	flat to slight	undulate to entire	slightly rough
3	5-12	irregular	flattish	undulate to lobate	rough pebbled

light tan-grey, with centre somewhat darker; some show white areas; pigment not elaborated into medium

may be grey, cream, or light brown with a grey exterior ring; pigment not elaborated into medium

tan-yellow with a white dot centre; none elaborated into medium

dirty-white; none elaborated into medium

yellow-tan centre with a greyish edge often evident; black centred forms will sometimes appear; none elaborated into medium

grey-white to whitish-yellow; none elaborated into medium

light yellow-brown to light grey; none elaborated into medium

dirty white-yellow with pale yellow or very pale green centre; some colonies dead white; none elaborated into medium

Isolate	Consistency	Effect on Agar	Opacity	Rough Numbers	Comments
1-3	fairly butyrous	usually depresses agar	translucent to opaque	several	
1-4	brown form generally butyrous, grey and cream forms somewhat drier	usually depresses agar	brown form translucent, grey and cream forms more opaque	many	brown form is a younger colony
1-6-2	a) butyrous	none or slight depression	opaque	several	these two morphological forms cannot be separated by purification procedures, are concluded to be morphological variants of the same organism
	b) slightly granular	none or slight depression	opaque	several	
1-8	butyrous	depression	young colonies are transparent or translucent, older colonies show translucent edge	several	same morphology as isolate 92
1-10	butyrous	none	opaque	many	
1-12-2	butyrous to slightly mucoid	none	opaque	few	
3	granular	none	opaque	few	morphologically similar to the <u>B. circulans</u> culture obtained from the Alberta Provincial Laboratory

Isolate	Consistency	Effect on Agar	Opacity	Rough Numbers	Comments
6	butyrous	none	opaque	many	occasionally shows a rougher form with more irregular edge and less creamy coloration
7-2	butyrous	none	opaque	many	
8-2	fairly butyrous	may show slight depression of agar	opaque with slightly translucent edge	few to several	
12-4	butyrous	none	opaque	many	
14-4	butyrous	none	opaque centre, with translucent or transparent edge	many	
19	butyrous	none	opaque	several	
22	butyrous	none	slightly translucent to opaque	many	
92	butyrous	depression	translucent particularly at the edge; younger colonies transparent	several	smooth white convex colonies on plate count agar

Table 2 (cont'd)

Isolate	Colonial Morphology					
	Diameter (mm)	Shape	Elevation	Edge	Surface	Pigmentation
130	1-3	circular	convex to umbilicate	usually entire	fairly smooth	pale lemon yellow becoming cream-yellow or light brown with age; none elaborated into medium
627	2	roughly circular to irregular	convex	undulate to slightly erose	fairly smooth	light grey to light yellow; often with small irregularly defined white flecks in centre; none elaborated into medium
629-3	1-1.5	slightly irregular	fairly convex	undulate	fairly smooth	light grey to light brown; none elaborated into medium
633	1	circular	convex	undulate	fairly smooth	translucent milky-white to a very light yellow-brown; none elaborated into medium
634	1-1.5	roughly circular	slightly umbilicate	undulate	fairly smooth	light grey to light tan; none elaborated into medium
635	1-1.5	circular	umbilicate	entire to slightly undulate	fairly smooth but somewhat rough in centre depression	light yellow-tan; none elaborated into medium
636	1-2	circular	convex	undulate	rough	light yellow-brown or grey-yellow; none elaborated into medium
12	1-2	circular	flat to slightly convex	entire	smooth	light grey; none elaborated into medium
<u>B. circ.</u>	4-8	roughly circular	flat	undulate to entire	rough but some white areas which are fairly smooth	greyish to dirty white with brighter white areas; none elaborated into medium

Isolate	Consistency	Effect on Agar	Opacity	Rough Numbers	Comments
130	butyrous	none	opaque but sometimes translucent	many	bright yellow convex colonies on plate count agar
627	slightly mucoid	none	opaque with translucent edge	many	
629-3	fairly butyrous	none	opaque	several	
633	butyrous to slightly granular	none	translucent	many	
634	butyrous	none	slightly translucent	many	
635	butyrous	often slight depression	opaque	many	
636	dry mucoid	none	opaque	several	
12	mucoid	none	slightly translucent to translucent	several	
<u>B. circ.</u>	butyrous	none	opaque	several	has lost most of the pebbled surface characteristic that it had when first acquired from the Alberta Provincial Laboratory

Table 2 (cont'd)

Isolate	Colonial Morphology					
	Diameter(mm.)	Shape	Elevation	Edge	Surface	Pigmentation
<u>B. myc.</u>	2-3 for those discernable as separate colonies; remainder spreading and filamentous	irregular or filamentous	flat over non-filamentous areas	filamentous; with single colonies erose, raised, or irregular	filamentous areas slightly rough; single colonies rough	grey-white or yellow-brown; none elaborated into medium
<u>B. subt.</u>	5-8	roughly circular to irregular	flat	irregular to erose	rough	white; none elaborated into medium

Isolate	Comments				
	Consistency	Effect on Agar	Opacity	Rough Numbers	
<u>B. myc.</u>	granular to slightly leathery; hard to emulsify in water	none	opaque	few but copious growth	
<u>B. subt.</u>	slightly granular; hard to emulsify in water	none	opaque	several	

nutrient broth plates, they have no difficulty in growing aerobically on this conventional media. Nor do they have any difficulty adapting to an iron reducing process if transferred from plate count to B10 broth. However, morphological differences between the colonies formed by the individual isolates are greatly accentuated on iron plate.

In particular, a depression of the agar surface in the centre of the colony often is observed for some of the iron reducers in B10 or B7 plate. Isolate 92, whose colonial morphology is illustrated by Plate 2, and isolate 1-8 show greater and more consistent agar depression than any of the other isolates. 1-3, 1-4, 1-6-2, 1-12-2, 8-2, and 635 may or may not show agar depression. Not all effective iron reducers show agar depression, the very effective isolate 130 (see Plate 3) being an example. However, any agar depressor found to date on a B7 or B10 plate has proved to be a strong reducer.

This agar depression is not a liquefaction of the agar. The reason for this depression is not known definitely, but it could represent a burrowing into the agar by organisms in the more anaerobic portions of the colony which are reducing iron and seeking a fresh source of Fe^{+++} . This could entail a certain degree of anaerobic digestion of the agar.

. This supposition is further borne out by the fact that 92 and 1-8 occasionally show on transfer from B10 broth to B10 plate a black area, presumed to be ferrous sulphide, in the centre of the depression. If 92 is transferred from Butlin's medium for sulphate



Plate 2. Three day colonies of isolate 92 showing agar depression on B10 plate. Magnification: 5X

Some of these depressions extend 2 mm. in depth into the agar.

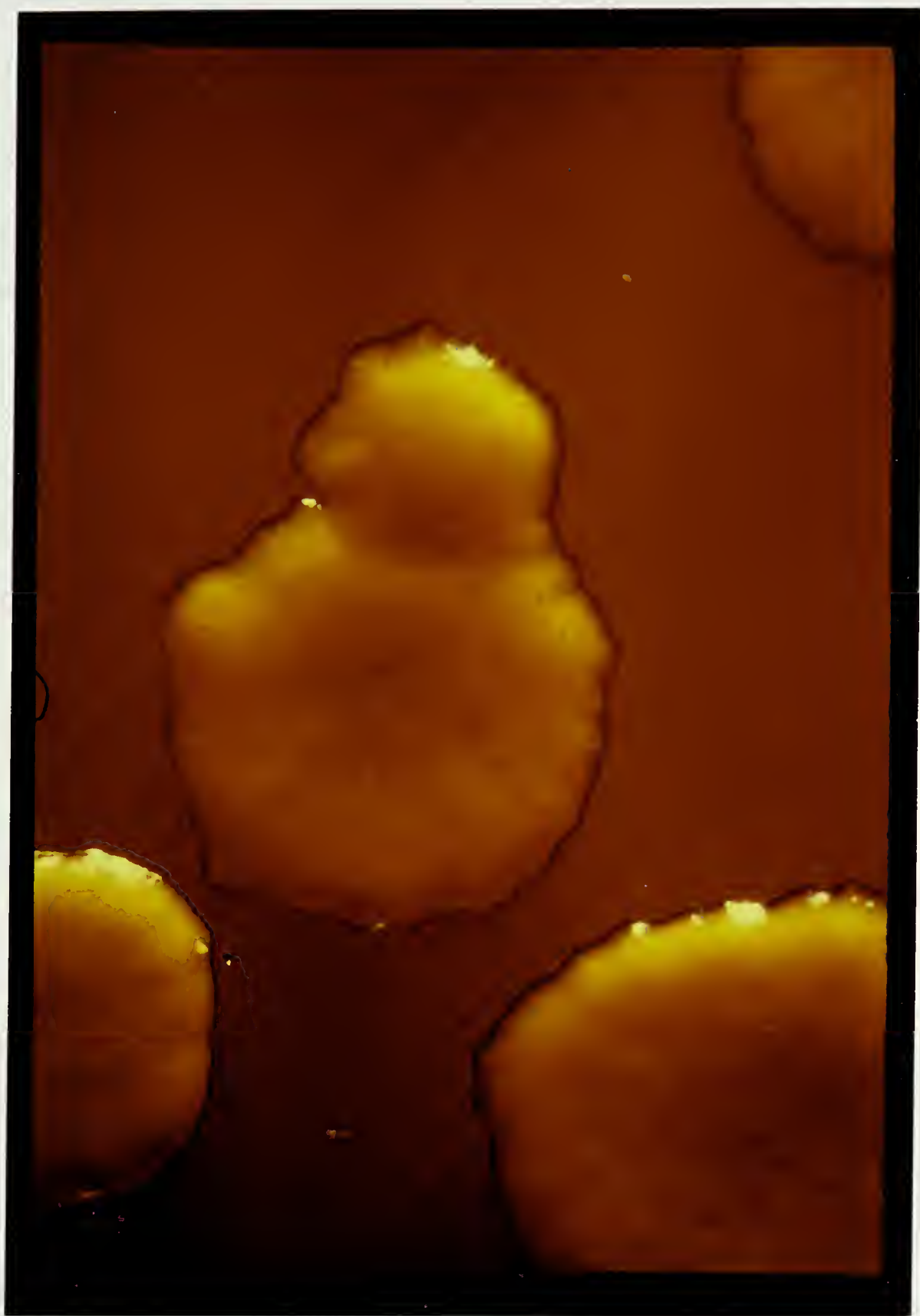


Plate 3. Three day colonies of isolate 130 on B10 plate.
Magnification: 20X

reducers amended with sulphite, black centres in the depressions are of regular occurrence.

At least three days of incubation on B10 plate are required before a colony of an effective iron reducer develops its distinguishing characteristics as opposed to the one day development of isolate 3 and the named Bacillus cultures, which are poor iron reducers. Many of the morphological differences between isolates listed in Table 2 admittedly appear to be minor. However, in most cases these slight differences have proved to recur with subsequent platings and some isolates forming colonies which appear to be similar upon a very superficial examination show quite dissimilar properties. Until further taxonomic work is done, these colonial differences form useful headmarks for distinguishing between these isolates.

Utilization of Simple Carbohydrates as Energy Sources

The use of some simple carbohydrates by iron reducing isolates as energy sources for reduction of Fe^{+++} under anaerobiosis is shown in Table 4. Only 92 and 1-8, which are similar organisms, utilize lactate and pyruvate successfully. Several isolates can utilize dextrose or sucrose. Succinate, acetate, and citrate, whose utilization in soil was found to be quantitatively parallel to iron reduction by Kumura et al (1963), cannot serve as substrates for most of our isolates. Only isolate 633 effectively reduces iron with citrate as the substrate, and none of the isolates use succinate or acetate very successfully. CO_2 production has been correlated with iron reduction

TABLE 4. Anaerobic utilization of simple carbohydrates as energy sources for growth and reduction of iron.

Isolate	Pyruvate	Succinate	Citrate	Acetate	Sucrose	Lactate	Dextrose
1-3	-	-	-	-	-	-	+++
1-4	-	-	-	-	-	-	+
1-6-2	-	-	-	-	-	-	-
1-8	+++	+	-	-	-	+++	++
1-10	-	-	-	-	++	-	+++
1-12-2	-	-	-	-	-	-	+++
3	-	-	-	-	+	-	+
6	-	-	-	-	+++	-	+++
7-2	+	-	-	-	-	-	-
8-2	-	-	-	-	-	-	++
12-4	-	-	-	-	+++	-	+++
14-4	-	-	-	-	-	-	+++
19	-	-	-	-	-	-	+++
22	-	-	-	-	-	-	+++
92	+++	++	-	++	++	+++	++
130	+	-	-	-	+++	+	-
627	-	-	-	-	+	+	++
629-3	-	-	-	-	+++	-	++
633	-	++	+++	++	+++	-	+++
634	-	-	-	-	+++	-	+
635	-	-	-	-	++	-	++
636	+	-	-	-	+++	-	+++
I2	-	-	-	-	-	-	-
<u>B.circ.</u>	-	-	-	-	-	-	+
<u>B.myc.</u>	-	-	-	-	-	-	++
<u>B.subt.</u>	-	-	-	+	-	-	+
Control	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-

Reduction:

+++ good
 ++ fair
 + slight
 - none

by Kumura et al (1963) and Takai et al (1963b). However, there was no gas production with any of our cultures. Either our selection of isolates is not very representative of the soil iron reducing population or the processes of citrate, acetate, and succinate utilization and CO₂ evolution are part of a process parallel to but not directly linked with the iron reduction process. More will be said later of the relationship of citrate metabolism and iron reduction.

Reduction of Nitrogen and Sulphur Compounds

Reduction of nitrogen and sulphur anions and ammonification by isolates are shown in Table 5. Most iron reducing isolates also reduce nitrate, and 627 and I2 are denitrifiers. None of the organisms can reduce sulphate in either Butlin's sulphate reducer medium or in S1 (sulphate) medium. 92 and 1-8 reduce sulphite to sulphide in both Butlin's + sulphite and S2 (sulphite) media. Thiosulphate is reduced by 92 and 1-8 much faster and more extensively than by the other few isolates which reduce this anion. The implication here is that several of these organisms, should they be able to utilize NO₃⁻, a sulphur compound, or Fe⁺⁺⁺ as a terminal electron acceptor, could therefore function through two or more of the four successive phases in waterlogging: 1) utilization of residual oxygen, 2) nitrate respiration, 3) iron reduction, and 4) sulphate reduction (Takai et al, 1963ab).

TABLE 5. Reduction of nitrogen and sulfur anions, growth in nutrient broth and ammonification in nutrient and B10 broths by iron reducing isolates.

Isolate	Nitrate Reduction		Reduction of Sulfur Compounds			Growth in Nutrient Broth	NH ₄ ⁺ Production	
	NO ₃ ⁻ → NO ₂ ⁻	NO ₃ ⁻ → Gas	SO ₄ ⁼ S ⁼	SO ₃ ⁼ S ⁼	S ₂ O ₃ ⁼ S ⁼		N.B.	B10
1-3	+	-	-	-	-	1	+	+
1-4	+	-	-	-	-	2	-	++
1-6-2	+	-	-	-	-	2	-	+++
1-8	+	-	-	+	+	3	+++	+++
1-10	-	-	-	-	-	2	-	-
1-12-2	+	-	-	-	-	2	-	+
3	+	-	-	-	-	4	-	+
6	-	-	-	-	sl.	3	-	-
7-2	+	-	-	-	-	3	-	-
8-2	+	-	-	-	-	2	-	+
12-4	+	-	-	-	fair	3	-	-
14-4	-	-	-	-	sl.	3	-	-
19	+	-	-	-	-	2	-	+
22	-	-	-	-	+	3	-	-
92	+	-	-	+	+	3	-	+++
130	-	-	-	-	sl.	1	-	-
627	→	+	-	-	-	1	-	+++
629-3	+	-	-	-	fair	3	-	-
633	+	-	-	-	fair	2	-	+
634	+	-	-	-	fair	3	-	-
635	+	-	-	-	+	4	-	-
636	+	-	-	-	fair	1	-	-
I2	→	+	-	-	-	1	-	-
<u>B.circ.</u>	+	-	-	-	-	4	-	+++
<u>B.myc.</u>	+	-	-	-	-	4	+++	+++
<u>B.subt.</u>	-	-	-	-	sl.	3	+++	+
Control	-	-	-	-	-	0	-	-

Growth:

NH₄⁺ Test:S⁼ Test:

0 none
 1 poor
 2 fair
 3 good
 4 very good

- NH₄⁺ absent
 + weak reaction
 ++ fair reaction
 +++ strong reaction

- no blackening
 sl. slight
 fair fair
 + good

Other tests: + or -
 → reduces NO₂

Ammonification by Isolates

Only half of the isolates grow well in nutrient broth and a strong ammonification process does not appear to be a feature of this class. Takai et al (1963b) linked strong ammonification and iron reduction but, as in the case of CO₂ evolution and the utilization of citrate, acetate, and succinate, the process of ammonification may be parallel to but not directly linked with the process of iron reduction.

Function of Isolates at High Temperature

All isolates except 92 can grow in B10 at 34°C. and reduce iron (Table 6). For three of the poorer reducers (3, B. circulans, and B. subtilis) reduction of iron was markedly increased at 34°C. Vitamin B12 had no effect in increasing the temperature tolerance of the reduction of iron, and no growth or reduction of iron was seen at 50°C.

Growth in Vitamin B12 denitrifier broth at high temperatures cannot be estimated, since a strange phenomenon occurs here. A granular white precipitate occurs in proportion to the usual quantity of growth in this medium at lower temperatures. Despite the fact that this precipitate does not appear in control, it is not considered to be growth but a side reaction of the inoculum.

Any action by isolates on NO₃⁻ at room temperature is duplicated at 34°C. In this case 92, which cannot grow in iron broth at 34°C., can do so in Vitamin B12 broth. 633 and B. circulans can reduce

TABLE 6. Reduction of Fe^{+++} and NO_3^- with increased temperature, and effect of Vitamin B12 on thermophilic Fe^{+++} reduction.

Isolate °C.	Medium																							
	B10								B10 + V. B12								V. B12							
	Growth				Fe ⁺⁺⁺ → Fe ⁺⁺				Growth				Fe ⁺⁺⁺ → Fe ⁺⁺				NO ₃ ⁻ → NO ₂ ⁻				NO ₃ ⁻ → Gas			
	25	34	50	65	25	34	50	65	34	50	65	34	50	65	25	34	50	65	25	34	50	65		
1-3	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
1-4	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
1-6-2	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
1-8	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
1-10	+	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-		
1-12-2	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
3	+	+	-	-	p	f	-	-	+	-	-	p	-	-	+	+	-	-	-	-	-	-		
6	+	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-		
7-2	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
8-2	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
12-4	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
14-4	+	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-		
19	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
22	+	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-		
92	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-		
130	+	+	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-		
627	+	+	-	-	+	+	-	-	+	-	-	f	-	-	→	→	-	-	+	+	-	-		
629-3	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
633	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	+	-	-	-	-	-		
634	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
635	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
636	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-		
I2	+	+	-	-	+	+	-	-	+	-	-	p	-	-	→	→	+	-	+	+	-	-		
B.circ.	+	+	-	-	p	f	-	-	+	-	-	f	-	-	+	+	+	-	-	-	-	-		
B.myc.	+	+	-	-	p	p	-	-	+	-	-	p	-	-	+	+	-	-	-	-	-	-		
B.subt.	+	+	-	-	p	f	-	-	+	-	-	f	-	-	-	-	-	-	-	-	-	-		
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		

Reduction of Fe^{+++} :

- negative
p poor
f fair
+ good

Nitrate Reduction:

+ or -
→ reduces NO_2^-

NO_3^- to NO_2^- at 50°C . Of the two denitrifiers, 627 is inactivated at 50°C . but I2, while it cannot denitrify at this temperature, does reduce NO_3^- to NO_2^- .

Rapidity with Which Fe^{+++} in B10 is Reduced

The similar isolates 92 and 1-8 show strong reduction in a tube of B10 broth much more quickly than the other isolates. 92 usually shows strong reduction within 16 hours, and 1-8 usually follows within 8 hours to half a day. Most of the remaining isolates require 2 to 3 days before showing strong reduction. 627 and 635 have a tendency to lag behind one day. 3, B. circulans, B. mycoides, and B. subtilis may show poor to fair reduction after 4 days, well after maximum growth has been reached.

Precipitates in B10 Broth

92 and 1-8 cultures usually decolorize as a white precipitate is formed. This precipitate was identified by X-ray diffraction as a hydrous ferrous phosphate. The only other culture regularly yielding a precipitate is 633. In this case the precipitate is a green gelatinous mass probably composed of ferrous hydroxide. This is the only pure culture that we have had which yields this green precipitate. However, it occurs regularly with primary isolations and is, in fact, usually a positive sign of a mixed or contaminated culture.

Isolates 1-8 and 92

Both isolates 1-8 and 92 are of particular interest because of

the extraordinary rapidity with which they reduce Fe^{+++} . Their characteristics are, for the most part, similar. Both exhibit the same colonial morphology, reduce NO_3^- to NO_2^- , and are unusual in that they are aerobic sporeformers which can reduce $\text{SO}_3^{=}$ to $\text{S}^{=}$. Of the selected isolates, only these two utilize lactate and pyruvate as energy sources. They are considered to be the same species, the only differences detected between them being that 1) the lake mud isolate (92) has a faster growth rate than the soil isolate (1-8) and 2) 1-8 can tolerate higher temperatures in iron medium than 92.

The Mechanism of Microbial Iron Reduction

Determination of Fe^{++}

Figure 2 shows the increase in Fe^{++} content of samples of B10 and 4 day cultures of isolates 92, 130, 14-4, and B. mycoides in the pH 3.5 acetate buffer plus o-phenanthroline. It appears that a two hour period is required for the o-phenanthroline to react with the Fe^{++} present in solution. This is distinctly different from the case of the standard curve samples, where color is developed immediately. It is thought, however, that the 2 hour reaction period, recommended in fact by Krishna Murti et al (1966) for their determination of Fe^{++} and Fe^{+++} , is necessary to liberate Fe^{++} from its association with the organic components of the medium. Krishna Murti et al note that the color for their determinations remained stable for at least 20 hours. In the case of determination of Fe^{++} it can be seen that optical density usually remains reasonably constant from two to

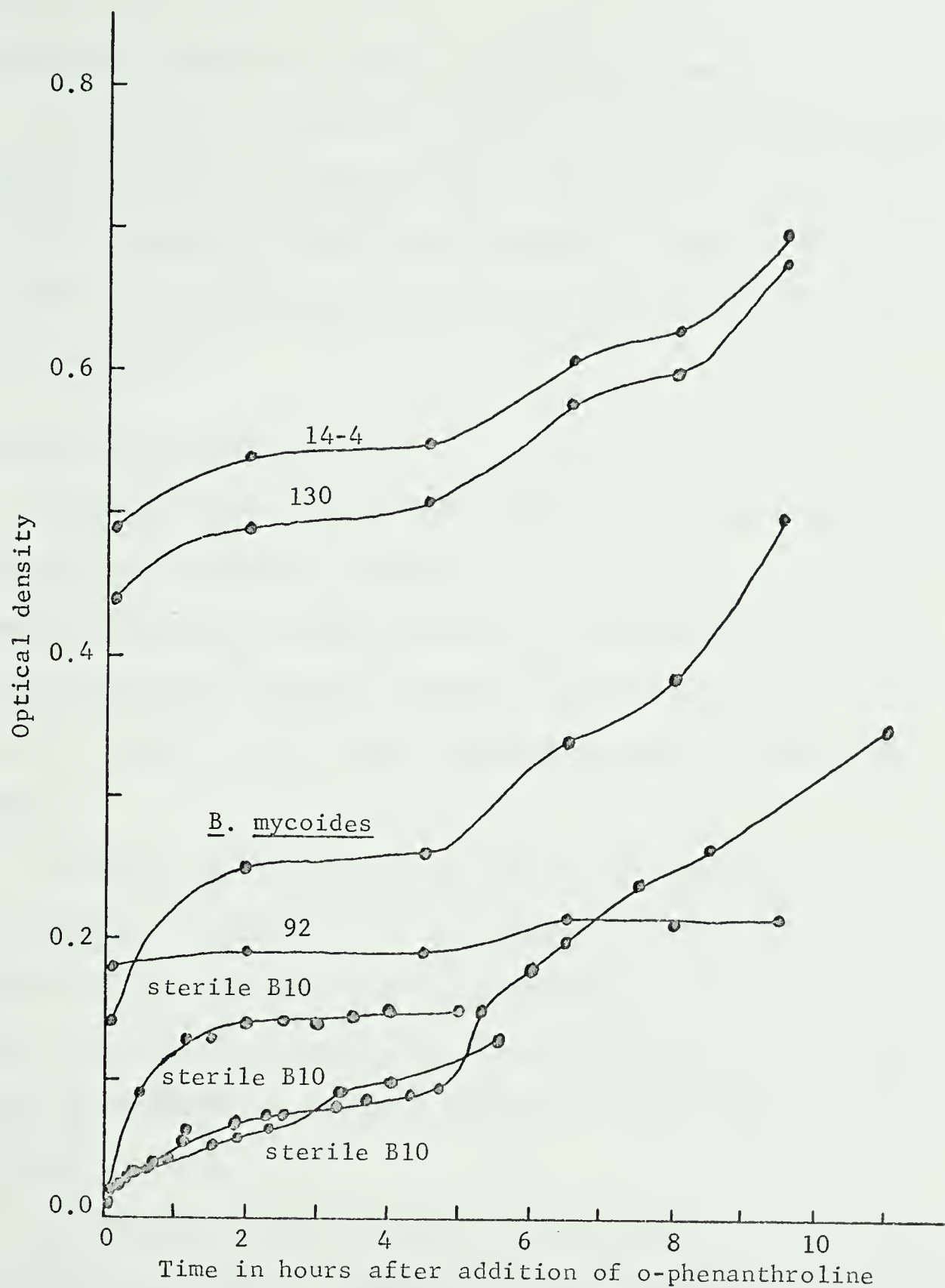


Figure 2. Increase in Fe⁺⁺ over time in samples from 4 day B10 cultures and uninoculated B10 medium while in 0.04% o-phenanthroline and 15%, 1 N, pH 3.5 Na-acetate-acetic acid buffer.

four hours, and then the reduction reaction proceeds.

It should be pointed out that the fact that autoredution was taking place was not realized until a major portion of the following work was completed. However, the procedure utilized to this point has been to take readings within two to four hours, so this work was not invalidated.

Incidental Reduction of Iron

Halvorson and Starkey (1927) felt that incidental reduction of iron, whereby anaerobic conditions and increase in acidity are created by microbial metabolism, plays an important role in soil. Not all organisms, however, can cause reduction of iron by reason of confluent growth, a truth well demonstrated during isolation procedures.

Reduction and growth in cultures of poor reducing isolates 3, B. circulans, B. mycoides, and B. subtilis decreased with anaerobiosis, indicating that reduction of Fe^{+++} is probably incidental in these cases. However, an increased degree of anaerobiosis had no effect, either in intensity or speed of reduction, on the other iron reducing isolates.

Isolate 92 was able to reduce iron strongly in a thin depth of B7 broth in a 250 ml. Erlenmeyer flask, a surprising result for conditions this aerobic. Rapid shaking on a rotary shaker was required to halt the process.

Decrease in pH, as Table 7 illustrates, does not play a role in the reduction of iron by these isolates. In fact, pH may increase

during the reduction process of the most effective isolates.

Table 7. pH of 4 day iron reducing cultures in B10 medium.

Isolate	pH	Isolate	pH	Isolate	pH	Isolate	pH
1-3	7.1	6	7.2	92	7.5	636	7.0
1-4	7.0	7-2	7.0	130	7.0	12	7.0
1-6-2	7.0	8-2	7.1	627	6.9	<u>B. circ.</u>	7.0
1-8	7.5	12-4	7.1	629-3	7.1	<u>B. myc.</u>	6.6
1-10	7.0	14-4	7.0	633	7.5	<u>B. subt.</u>	6.8
1-12-2	7.1	19	7.0	634	7.1	control	7.0
3	7.0	22	7.1	635	7.0		

An illustration of the effect of increased anaerobiosis and the behavior of pH and redox potential during the reduction of iron by isolate 92 is seen in Figure 3. Disappearance of Fe^{+++} was calculated by subtracting Fe^{++} from total Fe. pH had no effect on the reduction of iron. There was very little iron reduction due to anaerobiosis. The drop in redox potential was roughly parallel to the disappearance of Fe^{+++} and would be more likely to be controlled by the production of Fe^{++} rather than vice versa, since both curves show the characteristic sigmoid shape of a growth curve. Nearly complete reduction was assumed to occur; the increase in Fe^{+++} shown on the graph is due to precipitation of ferrous phosphate.

Table 8 illustrates the instability of the $\text{Fe}^{++}/(\text{Fe}^{++} + \text{Fe}^{+++})$ ratio in B10 broth which is only partly due to problems of Fe^{++} determination since even upon visual examination the intensity of the green

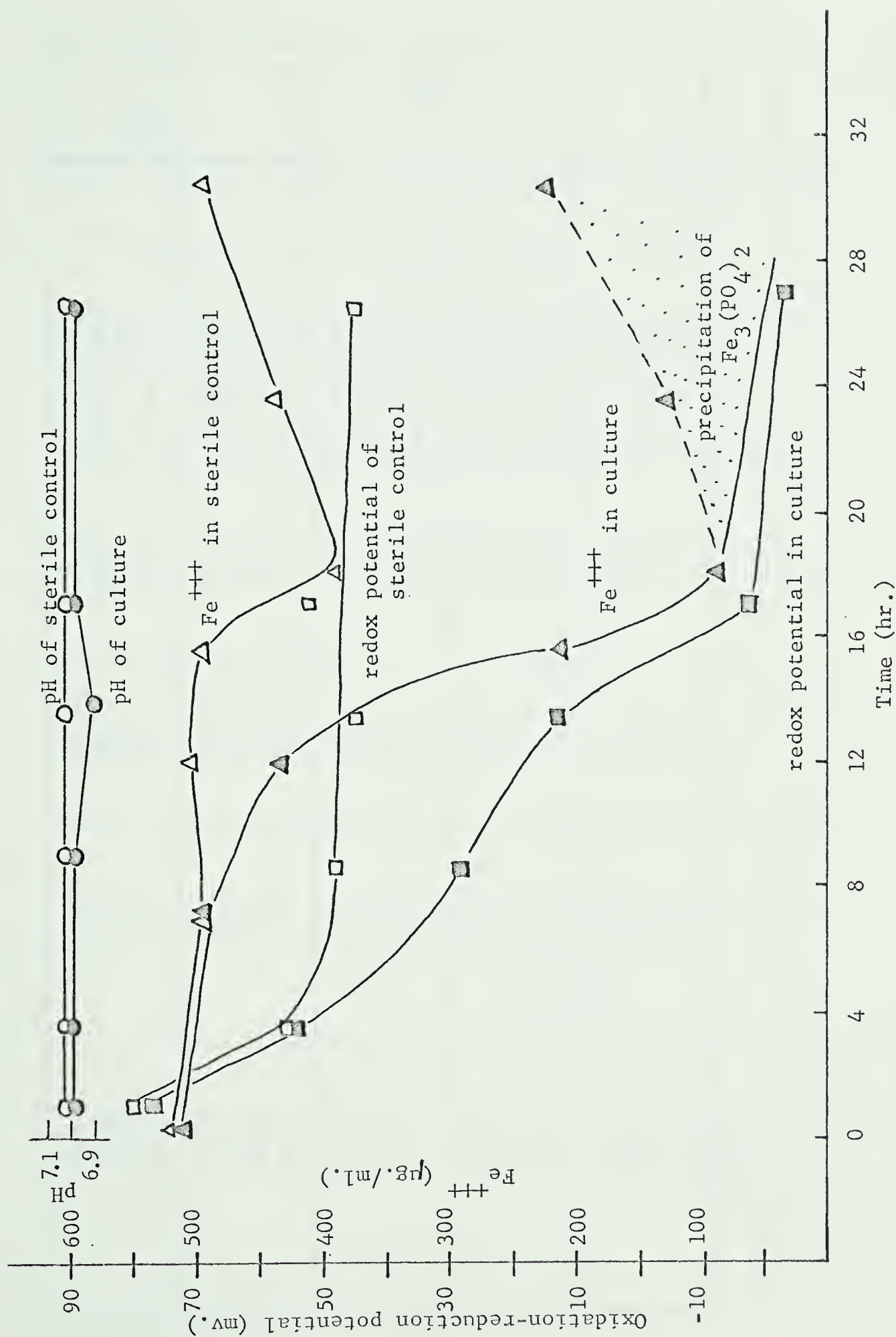


Figure 3. Relationship of pH and oxidation-reduction potential to the disappearance of Fe⁺⁺⁺ over time in an anaerobic culture of isolate 92 in B10 broth. Total [iron] at 0 hr. = 640 $\mu\text{g./ml.}$

TABLE 8. Effect of "cell-free" culture supernatants on the reduction of Fe^{+++} in B10 broth.

Isolate	Supernatant	Treatment	$[\text{Fe}^{+++}]$ in supernatant + B10 at 0 hrs. (mg./ml.)	$[\text{Fe}^{+++}]$ after 2 hrs. (mg./ml.)	Fe^{+++} reduced (mg./ml.)	Fe^{+++} reduced due to action of culture supernatant (mg./ml.)
Control	B10	not boiled	0.10	0.20	0.10	0.00
		boiled	0.11	0.20	0.09	0.00
	A3	not boiled boiled	0.05 0.05	0.09 0.11	0.04 0.06	0.00 0.00
92	B10	not boiled	0.07	0.16	0.09	-0.01
		boiled	0.08	0.15	0.07	-0.02
	A3	not boiled boiled	0.05 0.05	0.07 0.07	0.02 0.02	-0.02 -0.04
130	B10	not boiled	0.20	0.23	0.03	-0.07
		boiled	0.20	0.24	0.04	-0.05
	A3	not boiled boiled	0.05 0.05	0.07 0.07	0.02 0.02	-0.02 -0.04
14-4	B10	not boiled	0.20	0.24	0.04	-0.06
		boiled	0.20	0.23	0.03	-0.06
	A3	not boiled boiled	0.05 0.05	0.08 0.08	0.03 0.03	-0.01 -0.03
<u>B. myc.</u>	B10	not boiled	0.11	0.20	0.09	-0.01
		boiled	0.13	0.20	0.07	-0.02
	A3	not boiled boiled	0.05 0.05	0.07 0.08	0.02 0.03	-0.02 -0.03

color in the medium will vary unpredictably over the length of time in standing whether the medium is sealed or not. The medium will not, however, contain above 20% to 25% of the iron in ferrous form unless it has been stored for over two weeks in a sealed bottle. Even allowing for this instability, it is apparent that in none of these cultures (92, 120, 14-4, and B. mycoides) considered representative of our collection is there elaboration of extracellular enzymes or chemical products which will cause the intensity of reduction observed in a growing culture.

Incidental reduction of iron, therefore, does not appear to be of importance in the case of the iron-reducing isolates.

The Role of Iron Reduction in the Anaerobic Cell

The use of Fe^{+++} as a terminal electron acceptor:

Table 9 illustrates the benefit derived by the iron reducing isolates from Fe^{+++} under anaerobiosis. These results are from a run where the tubes of inoculated media were agar-sealed, and are estimated visually. The first hypothesis resulting from initial experiments of this type was a division of isolates into two classes: obligate and incidental iron reducers (Cook et al, 1966). The incidental reducers apparently could grow as well without as with Fe^{+++} under anaerobiosis and therefore, it was thought, the reduction of iron was incidental to the metabolism of the cell. The obligate reducers apparently require Fe^{+++} under anaerobiosis since they cannot grow in A3 without oxygen.

TABLE 9. Visual estimation of anaerobic growth of iron-reducing isolates in B10 and control A3 broths.

Isolate	Age of Culture (days)							
	1 1/2		2		3		4	
	B10	A3	B10	A3	B10	A3	B10	A3
1-3			2R	tr	4	2		
1-4			2R	tr	3	1		
1-6-2					3R	tr		
1-8	2R	0			3	tr		
1-10			1R	tr	3	1		
1-12-2			3R	1			4	1
3							1	1
6					3R	1		
7-2					3R	2		
8-2			4R	2	4	3		
12-4					3R	3	4	3
14-4					4R	4		
19					4R	3		
22			2R	1	4	3		
92	3R	0			3	tr		
130	3R	1			3	1	4	2
627							2R	0
629-3					3R	3	4	3
633					4R	1	4	2
634			4R	1	4	2	4	3
635			3R	1	4	2		
636			3R	0	3	tr	4	1
I2							2R	1
<u>B.circ.</u>							tr	tr
<u>B.myc.</u>							tr	tr
<u>B.subt.</u>							1	tr
Control	0	0	0	0	0	0	0	0

Growth:

0 none
tr trace
1 light
2 fair
3 good
4 heavy

Reduction:

R first appearance of
strong reduction

Since all of the isolates are capable of aerobic growth in A3, the function of Fe^{+++} under anaerobiosis is likely to be that of a terminal electron acceptor which is reduced in lieu of oxygen, a process possibly analogous to nitrate and sulphate reductions. The function of the high quantity of iron utilized in B7 or B10 is not likely as a nutrient since there is enough iron in A3 to amply fulfill micronutrient requirements and support heavy aerobic growth.

This interpretation is valid for the "obligate" iron reducers. However, closer examination of the "incidentals" showed that these also utilized iron as an electron acceptor. All except isolate 14-4 upon visual examination showed inferior growth in A3 to that in B10. The quantity of growth in A3 compared to B10 varied between isolates, with no definite delineation between "classes". It is likely that the difference is due to the efficiency of the organism in utilizing organic compounds in the medium as electron acceptors. An organism such as 92 apparently cannot utilize organic acceptors with the same success as 130, and 130 is not as successful as 14-4 under anaerobiosis in A3. B. mycoides and the other poor reducers of iron essentially grow equally poorly in either medium, with less reduction under anaerobiosis than with their copious aerobic growth. The reason for reduction by these organisms is not known, since culture supernatants of B. mycoides did not reduce appreciable quantities of Fe^{+++} in B10.

Isolate 14-4 upon visual inspection appears to grow as well under anaerobiosis in both A3 and B10. A plate count study of 4

day cultures, however (Table 10), show double the growth in B10 compared to A3. Other experiments with organisms growing well in A3 have verified the beneficial effect of Fe^{+++} under anaerobiosis (Cook et al, 1966) and even in these cases Fe^{+++} acts as a terminal electron acceptor.

Table 10. Beneficial effect of Fe^{+++} under anaerobiosis on the growth of isolate 14-4.

Medium	Visual Estimation of Growth	Population on Plate Count Agar	Fe^{+++} Reduction
B10	4	150×10^5	+
A3	4	76×10^5	

Extent of reduction by representative isolates:

The results of measuring the extent of reduction in B10 broth by 92, the "obligate" reducer; 130, which shows fair growth anaerobically in A3; 14-4, with good growth in A3; and B. mycoides, the poor reducer, are shown in Table 11.

Table 11. Percentage of iron reduced in 4 day B10 cultures of representative iron reducing isolates.

Isolate	% Reduction
Sterile B10 Control	26
92	100
130	70
14-4	77
<u>B. myc.</u>	35

The high background of Fe^{++} in sterile B10 is noticeable, as well as the weak reduction by B. mycoides. By visual inspection the reduction in this particular B10 culture of B. mycoides would have been termed fair. 130 and 14-4 show strong reduction, with essentially no difference. Their reduction is typical of most of the isolates, which do not carry the reduction to completion. 92 and 1-8, and occasionally a few of the other cultures, carry the reduction almost, if not entirely, to completion. In this case the 100% reduction is assumed, since a ferrous phosphate precipitate prevented determination. However, Figure 3 shows at least a 90% reduction in an anaerobic jar of a B10 culture of isolate 92 before precipitation began.

The use of NO_3^- and $\text{SO}_3^{=}$ as terminal electron acceptors by isolate 92:

Table 12 portrays the results of the experiment to determine whether isolate 92 utilizes NO_3^- and $\text{SO}_3^{=}$ as terminal electron acceptors.

The control media (Vitamin B12m and Butlin's with no $\text{SO}_3^{=}$) were good media for aerobic growth. (Reductions of NO_3^- and $\text{SO}_3^{=}$ in unsealed controls take place in the anaerobic depths of these tubes.) $\text{SO}_3^{=}$ definitely appears to act as a terminal electron acceptor since there is no anaerobic growth in control. Despite the lesser growth in Vitamin B12 compared to Vitamin B12m, it is certain that NO_3^- is used as an electron acceptor since 1) there is poor growth in control Vitamin B12m, 2) strong production of NO_2^- does occur, and 3) in a tube of Vitamin B12 plus B10 nitrate reduction occurs with isolate 92 but

not Fe^{+++} reduction (Chapter III). In this last case NO_2^- was shown to be toxic to isolate 92, and the same is true for this experiment. In soil this would pose no problem to 92 since other organisms would carry on the detoxifying reaction of reduction of NO_2^- . Isolate 92, therefore, should be able to function during all four of the phases of waterlogging in soil.

Table 12. Utilization of NO_3^- and $\text{SO}_3^{=}$ as terminal electron acceptors by isolate 92 (3 day cultures).

Condition	Medium			
	Vitamin B12 Growth $\text{NO}_3^- \rightarrow \text{NO}_2^-$	Growth in Vitamin B12m	Butlin's+ $\text{SO}_3^{=}$ Growth $\text{SO}_3^{=} \rightarrow \text{S}^{=}$	Growth in Butlin's (no $\text{SO}_3^{=}$)
Unsealed tube	4 +	4	4 +	4
Agar- sealed tube	tr +	1	3 +	0

Visual estimation of growth:

4 heavy
3 good
1 light
0 none

Reduction of iron as a growth-linked process:

Figure 4 illustrates that the reduction of iron by isolate 92 under anaerobiasis in B7 broth is growth-linked. The experiment in B10 broth (Figure 5) provides supporting evidence, although the plate count results were less satisfactory than in the first run. Fe^{+++} was not reduced in the sterile B10 controls. The results in Figure 5

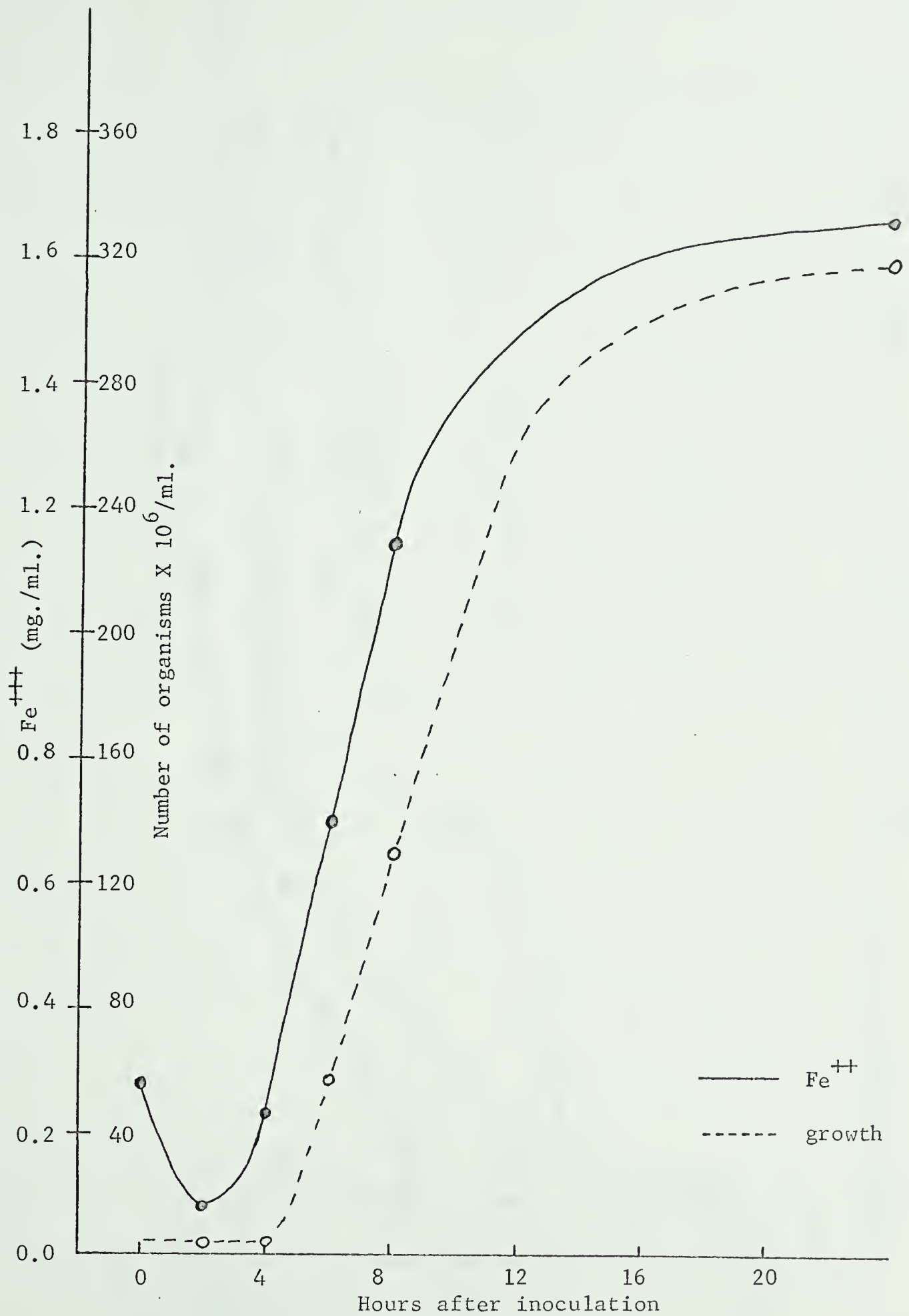


Figure 4. Relation of growth and reduction in a B7 culture of isolate 92. Total [iron] in B7 = 1.75 mg./ml.

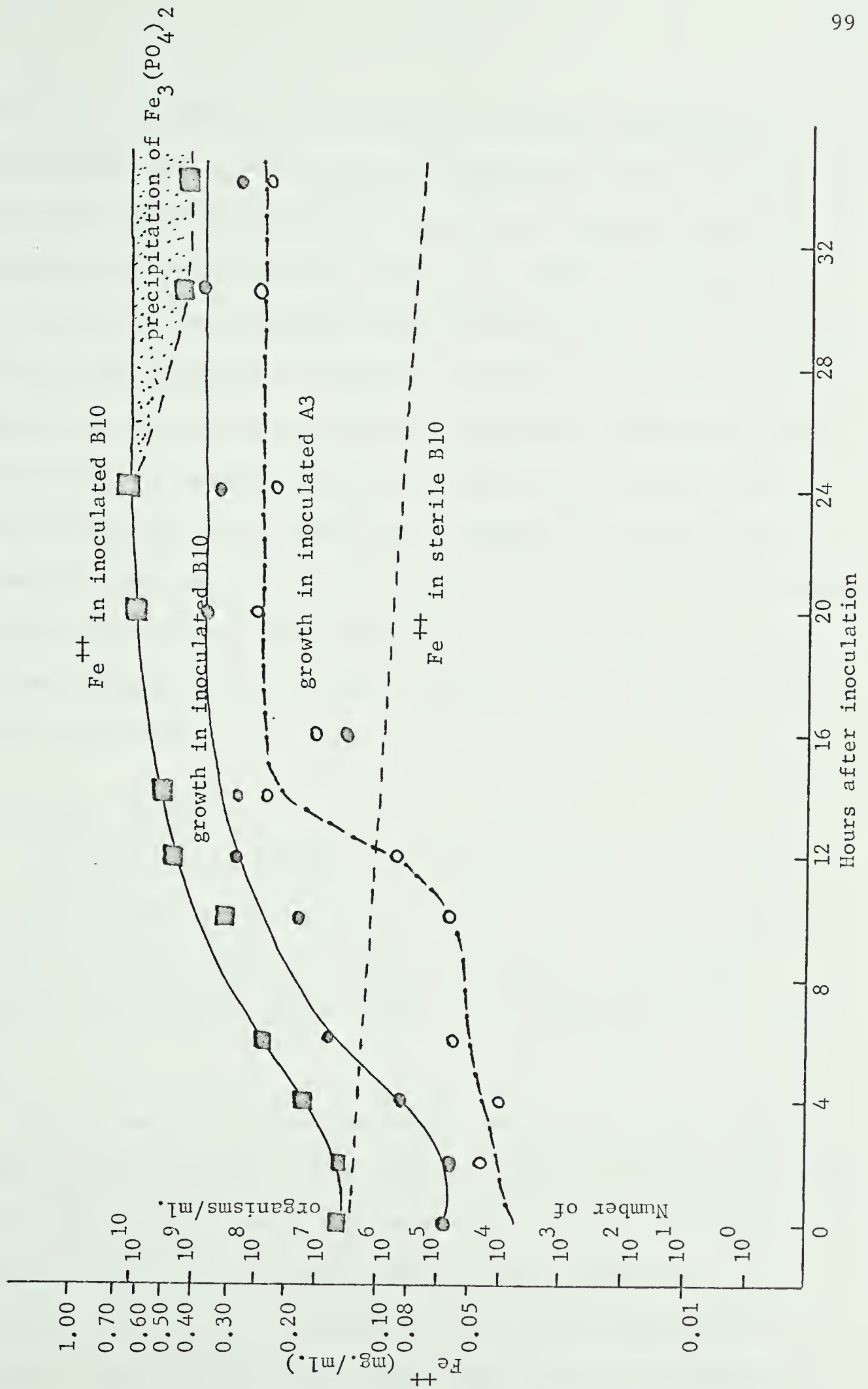


Figure 5. Relation of growth and reduction in a B10 culture of isolate 92 and comparison of anaerobic growth with and without Fe⁺⁺. Total [iron] in B10 = 0.66 mg./ml.

were plotted logarithmically in order to compare the growth of isolate 92 in B10 and A3. A growth curve is evident for A3 but it is apparent that without Fe^{+++} the lag phase is prolonged and only a tenth of the growth of the B10 culture occurs in A3 under anaerobiosis.

Efforts to duplicate the growth and reduction curves utilizing the apparatus of Figure 1 and CaCO_3 as a CO_2 source were unsuccessful. The reduction curve was obtained successfully but difficulties were encountered in obtaining duplicate counts on plate count. This is thought to be due to the inability of isolate 92 to adapt readily to aerobic conditions after very strict anaerobiosis, a fact which has been observed at other times for this isolate and others. There is, however, ready adaptation to the reversed condition, which will be illustrated later.

Resting cell studies:

Resting cells of isolates 1-8 and 92 reduced Fe^{+++} in B19-pyruvate and B19-lactate resting cell media after 12 hours, while boiled cells had no effect. This was considered to be further proof that reduction of Fe^{+++} is due to the metabolism of the living cell.

At the end of 1 day of incubation, none of the other isolates showed positive response. Some isolates showed strong reduction after 3 to 5 days, but this could have represented growth at the expense of dead cell constituents. Considering the small quantities of cells which were used, and the difficulties which can be encountered in obtaining viable resting cells, these results are not considered to

have disproved the contention that Fe^{+++} is used as an electron acceptor.

The first experiment to estimate the stoichiometric relationship of lactate oxidized and Fe^{+++} reduced yielded only quantitative results. Cells in the higher concentration reduced Fe^{+++} more quickly than in lower concentration. Boiled cells had no effect. Lactate could not be estimated due to "background" material, presumably of lysed cells, which yielded color development too intense to be read spectrophotometrically. Also, standard curves that resulted were very poor.

Contamination of the sample was thought to be a problem and efforts were made to "desensitize" the H_2SO_4 reaction by adding the H_2SO_4 to samples incubated at higher temperatures. If, by lowering the increase in optical density per unit increase in lactate, the range of the lactate determination could be broadened then the effect of contamination would be decreased. When H_2SO_4 was syringed on standards incubated at 50°C . and 65°C ., very erratic results were obtained. However, good curves were obtained (Appendix III) from the room temperature and 50°C . standards. The 50°C . standards gave a much more violent reaction with H_2SO_4 than those at room temperature, and the curve was desensitized, probably indicating that the oxidation had gone past acetaldehyde. However, there was not enough desensitization to effectively broaden the range of determination.

The greatest source of error was concluded to be due to the fact that the concentrated H_2SO_4 must be syringed on the sample, at best a variable procedure which often leads to incomplete oxidation of

lactate if insufficient heat is developed. The use of triplicate determinations on samples at room temperature was the procedure adopted.

A second resting cell experiment utilizing the new B20 r.c. medium still yielded optical densities beyond the range of the lactate standard curves. The results of the final experiment, where standard curves for lactate were performed with the cells present, were within range but there was obvious "background" in the cultures. With time, "lactate" concentration in the iron-free control for endogenous metabolism nearly doubled, probably due to lysis of cells. The Fe^{+++} reduction curve which resulted was unmistakably a growth curve with lag and logarithmic phases. Surviving cells were growing, probably, at the expense of the dead. A very rough correlation was seen between iron reduction and lactate utilization (Figure 6) but no stoichiometric relationship could be deduced.

If further work with resting cells is contemplated, more refinement of technique is required. First, the resting cell system requires increased buffering to reduce lysis of cells. That pyruvate can be utilized as an energy source was a recent finding and future work may centre on pyruvate utilization rather than lactate, thereby omitting the lactate to pyruvate step. Enzymatic rather than chemical determinations may be employed, although a chemical method would be preferred when the substrate is in relatively large quantity and several determinations must be made. Possibly resting cell work should be omitted in favor of work with crushed cell fractions.

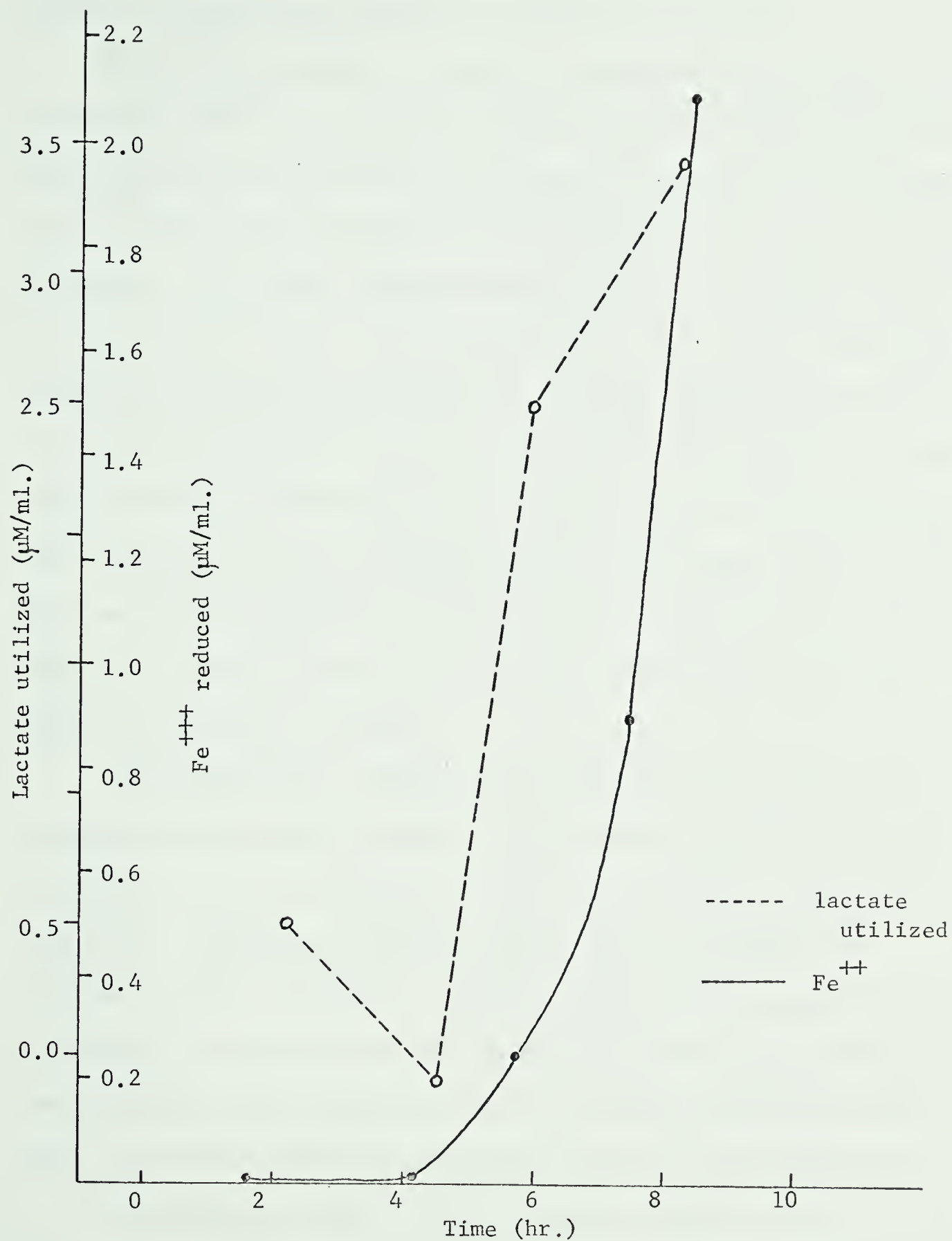


Figure 6. Fe^{+++} reduced and lactate utilized by isolate 92 in B20 resting cell medium.

Effect of respiratory inhibitors on reduction of Fe^{+++} :

The effect of cyanide, azide, and carbon monoxide on the reduction of Fe^{+++} by the iron reducing isolates is shown in Table 13. 10^{-4} M and 10^{-3} M CN^- and 10^{-4} M N_3^- have no effect on the reduction of Fe^{+++} due to the masking effect of iron (Hochster and Quastel, 1965). Similarly, all isolates showed copious growth after aerobic incubation in B10 with 10^{-3} M KCN. 10^{-2} M CN^- and 10^{-3} M N_3^- either diminished, delayed, or halted Fe^{+++} reduction. Considering the high concentrations of these inhibitors and the fact that their action in the cell is not entirely specific for the cytochrome system, the possibility that reduction of iron was halted due to other harmful effects on the cell was noted. However, in most cases where reduction was inhibited there was growth in those cultures which normally show anaerobic growth in A3 control medium.

Thus these results implicate the involvement of a cytochrome-linked electron transfer chain in the reduction of Fe^{+++} which could be analogous in many respects to that of NO_3^- and $\text{SO}_4^{=}$ respiratory reduction. Bromfield's (1954b) results with B. circulans were contrary to this conclusion. However, these results corroborate the findings of Jamanaka and Motomura (1959) and Kumura et al (1963), who had postulated that reduction of Fe^{+++} was due to microbial action when respiratory inhibitors halted the process in waterlogged soil.

The inhibitory effect of carbon monoxide (Table 13) is masked by iron (Hochster and Quastel, 1965). However, it is of note that definite inhibition of iron reduction was

Table 13. Effect of respiratory inhibitors on the reduction of Fe^{+++} in B10 culture.

Isolate	Cyanide (M)			Control, NOCN^- or N_3^-	Azide (M)			Carbon Monoxide	
	10^{-4}	10^{-3}	10^{-2}		10^{-4}	10^{-3}	10^{-2}	Control, no CO	CO
1-3	+++ (2)	+++ (3)	—	+++ (2)	+++ (3)	+++ (6)	—	++	+++
1-4	+++ (3)	+++ (3)	—	+++ (2)	+++ (2)	+++ (3)	—	+++	+++
1-6-2	+++ (4)	+++ (3)	—	+++ (2)	+++ (2)	+++ (3)	—	+++	+++
1-8								+++	+
1-10	+++ (5)	+++ (6)	+(6)	+++ (3)	+++ (2)	—	—	+++	+++
1-12-2	+++ (2)	+++ (4)	—	+++ (2)	+++ (3)	+++ (6)	—	+++	+++
3	+(6)	+(6)	—	+(6)	—	—	—	+	+
6	+++ (3)	+++ (3)	+(6)	+++ (3)	+++ (2)	—	—	+++	+++
7-2	+++ (3)	+++ (3)	++ (6)	+++ (2)	+++ (3)	—	—	+++	+++
8-2	+++ (3)	+++ (4)	—	+++ (2)	+++ (3)	+++ (6)	—	+++	+++
12-4	+++ (3)	+++ (3)	—	+++ (3)	+++ (3)	—	—	+++	+++
14-4	+++ (3)	+++ (3)	+++ (5)	+++ (3)	+++ (3)	—	—	+++	+++
19	+(6)	—	—	++ (6)	+++ (3)	+++ (4)	—	+++	+++
22	+++ (3)	+++ (2)	+++ (5)	+++ (3)	+++ (3)	+(6)	—	+++	+++
92	+++ (3)	+++ (3)	—	+++ (2)	+++ (2)	—	—	+++	+
130	+++ (3)	+++ (2)	+++ (6)	+++ (2)	+++ (2)	++ (6)	—	+++	+++
627	+++ (6)	++ (6)	—	+++ (6)	+++ (6)	—	—	++	—
629-3	+++ (2)	+++ (3)	+++ (6)	++ (2)	+++ (2)	—	—	+++	+++
633	+++ (3)	+++ (3)	+++ (6)	+++ (3)	+++ (4)	—	—	+++	+++
634	+++ (4)	+++ (2)	++ (6)	+++ (3)	+++ (2)	+(6)	—	+++	+++
635	+++ (4)	+++ (4)	—	+++ (4)	+++ (3)	—	—	+++	++
636	+++ (4)	+++ (6)	—	+++ (6)	—	—	—	+++	+++
I2	+++ (3)	+++ (6)	—	+++ (2)	+++ (4)	+++ (6)	—	+++	+
<u>B. circ.</u>	+(6)	—	—	+(6)	—	—	—	+	—
<u>B. myc.</u>	—	—	—	—	—	—	—	+	—
<u>B. subt.</u>	+(6)	—	—	—	—	—	—	+	—
Control	—	—	—	—	—	—	—	—	—

Visual estimation of reduction:

Speed of reduction:

+++ strong

++ fair

+ weak

— none

(3) 3 days

seen for the only organisms which utilize $\text{SO}_3^{=}$ as a terminal electron acceptor (isolates 92 and 1-8) and for the only organisms which are denitrifiers (isolates 627 and I2). The involvement of the cytochrome system for these organisms at least is strongly suggested. It is probable that the existence of an "iron reductase" enzyme similar to cytochrome oxidase or nitrate reductase can be postulated.

"Iron Reductase" as a Constitutive Enzyme of Isolate 92

Figure 7 illustrates that a culture of isolate 92 grown aerobically in A3 for several transfers requires no more adaptation time before reducing Fe^{+++} than if the culture was grown anaerobically in B10. For this reason the "iron reductase" postulated as the enzyme responsible for reducing Fe^{+++} is considered to be a constitutive enzyme.

Aspects of the Ecology and Function of Iron Reducing Organisms in Soil

The Effect of Various Environmental Factors on the Population of Iron Reducers in Soil

Estimation of population by Most Probable Number technique:

The population of iron reducing bacteria in soil has been estimated by MPN technique. Unsealed tubes of B7 or B10 medium were used and visible reduction after one week considered a positive result. Five days to one week was the incubation period required at room temperature to obtain maximum numbers. There was little reduction

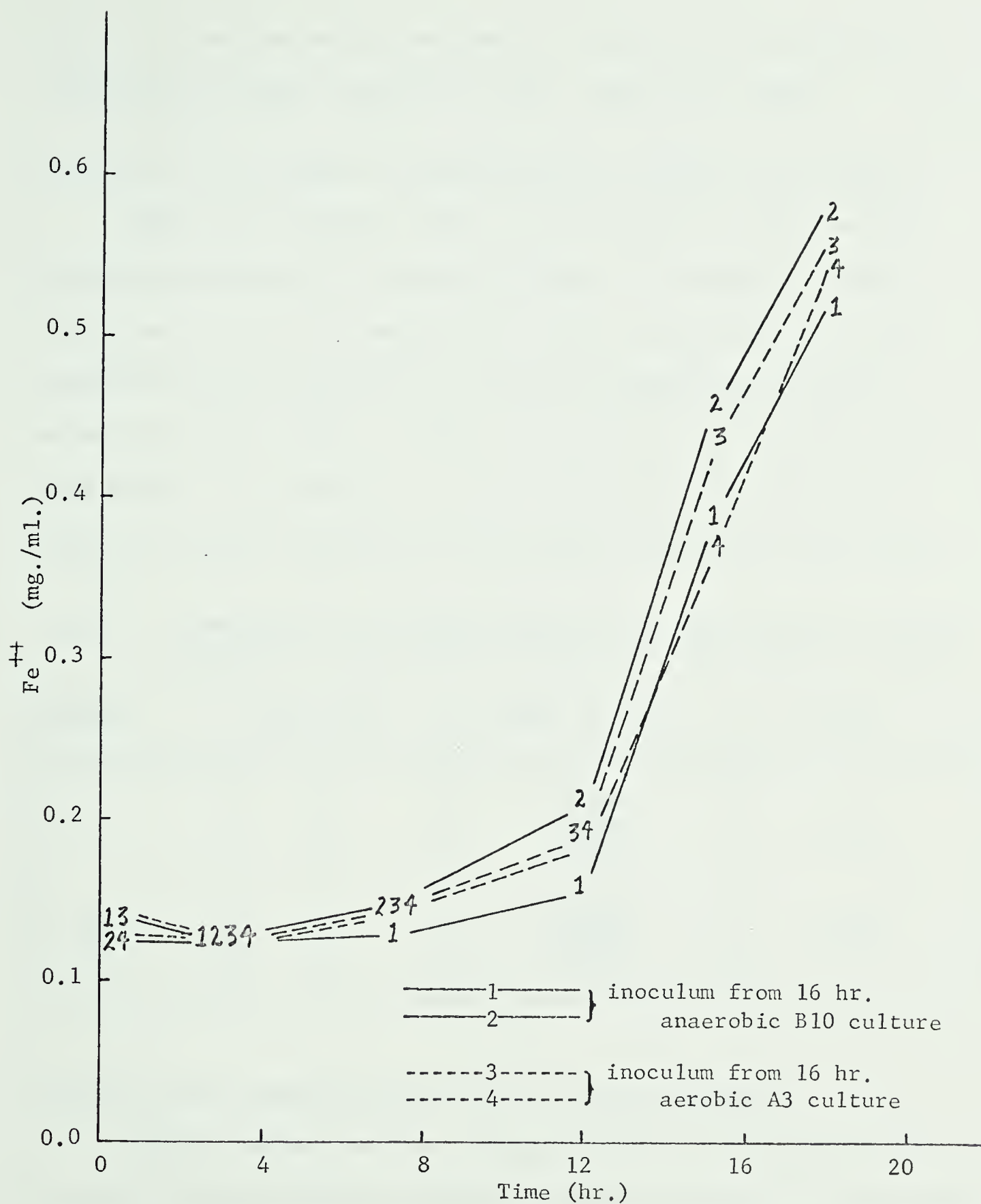


Figure 7. Adaptation of isolate 92 from aerobic conditions in A3 to the anaerobic Fe^{+++} -reducing process in B10.

after this time and counts were made at the end of one week since reoxidation of some cultures will occur after this period.

Comparison of iron media for determination of MPN of iron reducers:

Many of our earlier counts of iron reducers in soil by MPN technique utilized B7 as the growth medium. To determine whether these results could be compared with others using B10 medium, the population of iron reducers in a black chernozemic Ah sample was enumerated in both media. Table 14 shows that 1) replication within each medium is satisfactory for this type of determination and 2) there is no great difference in the numbers obtained by each medium.

Table 14. Comparison of B7 (ferric ammonium citrate) and B10 (ferric phosphate) media for enumeration of iron reducers in soil.

Medium	Replicate	MPN/g. of soil	Average
B7	1	16,000	16,000
	2	9,000	
	3	22,000	
B10	1	22,000	27,000
	2	35,000	
	3	24,000	

Survey of iron reducing populations in various habitats:

Table 15 shows the iron reducing population of several soils. The fact that iron reducing organisms are non-specific for any certain soil, as Daragan (1966) points out, is well borne out here. The numbers agree well with Roberts (1947) but are only a tenth of

Table 15. Survey of iron reducing populations in various habitats.

Sample No.	Description	General Location	Sampling Date	Medium Used
17	Orthic Black Chernozem Ah	Edmonton area	May/'65	B7
18	Ah			
19	Bm			
20	C			
21	Orthic Black Chernozem Ah	Edmonton area	May/'65	B7
22	Ah			
23	Bm			
24	C			
25	Humic Gleysol Ahg	Edmonton area	May/'65	B7
26	Bg			
29	Orthic Grey Wooded F	Edmonton area	May/'65	B7
30	Ae			
31	AB			
32	Bt			
33	Ck			
35	Orthic Grey Wooded Ac	Edmonton area	May/'65	B7
36	AB			
37	Bt			
38	Ck			

Sample No.	Depth of Sample (ft.)	Cultivation	% Moisture (O.D. basis)	MPN X 10 ³ per O.D. g. of soil
17	0.3	—	31.1	4.5
18	1.0	—	28.0	0.35
19	1.5	—	15.2	0.28
20	3.0	—	16.4	0.23
21	0.5	+	29.3	12
22	1.2	+	26.5	0.54
23	2.0	+	13.1	0.19
24	2.8	+	13.4	0.52
25	0.3	—	230	98
26	0.8	—	22.2	1.8
29	0.2	—	79.8	97
30	0.3	—	16.7	18
31	0.5	—	18.3	0.54
32	0.8	—	20.0	0.51
33	4.0	—	17.3	0.02
35	0.5	+	10.6	47
36	0.8	+	12.3	1.4
37	0.9	+	20.0	1.3
38	2.0	+	17.0	0.53

Table 15. (cont'd.)

Sample No.	Description	General Location	Sampling Date	Medium Used
39	Orthic Dark Grey Wooded Ahe	Edmonton area	May/'65	B7
40	AB			
41	Bt			
42	Ck			
43	Orthic Dark Grey Wooded Ahe	Edmonton area	May/'65	B7
44	AB			
45	Bt			
46	BC			
60	Minimal Podzol* LH	Whitecourt area	Aug./'66	B10
61	Ae			
62	Bf			
63	C			
—	Dark Grey Solod Ahe	Beaverlodge Research Station	July/'66	B10
—	Dark Grey Solod Ahe			
—	Lawn Soil	Grande Prairie	July/'66	B10
—	Coarse undecomposed surface layer of bog	Fox Creek	July/'66	B10
—	Portion of beaver dam below water level			
—	Iron oxide coated streambed downstream from beaver dam			

* Collected and classified by J. Dangerfield.

Sample No.	Depth of Sample (ft.)	Cultivation	%OM.	pH	% Moisture (O.D. basis)	MPN X 10 ³ per O.D. g. of soil
39	0.5	—			22.5	8.6
40	1.0	—			18.6	2.0
41		—			20.0	1.4
42		—			24.0	0.43
43	0.5	+			23.6	49
44	1.0	+			16.9	16
45	1.2	+			16.1	0.19
46	2.0	+			14.0	0.15
60		—	67.8	5.9	81.8	45
61		—	2.1	6.3	10.3	22
62		—	0.9	5.6	7.9	2.6
63		—		5.8	3.5	0.83
—	0.3	—			39.2	82
—	0.3	+			21.9	52
—					23.8	110
—					500	27
—					550	660
—					150	17

Daragan's. However, Daragan looked for a positive test with α - α' dipyridal, which can be less demanding than the visual evidence of strong reduction which was taken as the standard for these results. Also, it should be pointed out that his medium was not as rich as B7 or B10.

These results indicate that iron reducers compose a consistent but not a large segment of the bacterial population of soil, if 10^8 bacteria per gram (Alexander, 1961) is taken as the bacterial population of a fertile A horizon. They are most numerous in the surface horizons of mineral soils but the figures given for mineral A horizons are, as Gardner (1967) points out, only 1% of the population of organic soils.

It is apparent that increased organic matter and increased moisture benefit these organisms. No conclusion could be drawn from comparing the figures for paired cultivated and uncultivated samples of soil types as to which condition was more favorable.

The effect of soil properties on the population of iron reducers:

Table 16 compares several soil properties with the population of iron reducers. The samples in the field were very dry, which essentially had the effect of holding the moisture factor constant. The numbers are lower overall for A horizons than in Table 15 due to the dry conditions, but a good proportion of the population is present, presumably due to the iron reducer's sporeforming ability. Organic matter content is seen to be a major factor influencing the iron

Table 16. Effect of soil properties on the population of iron reducers.

Sample No.	Soil	Horizon	Sample depth(ft.)	% Moisture	MPN X 10 ³ per g. O.D. soil
64	Orthic Black Chernozem	Ah	1.5	7.4	1.9
65		Bm	2.5	4.4	0.1
66		Ck	4.5	6.3	0.06
67	Black Solod	Ah	0.5	3.7	2.8
68		Ae	1.0	1.2	0.3
69		Bnt	2.5	9.4	0.6
70		Ck	3.5	8.5	0.05
71	Eluviated Black	Ah	1.0	2.8	1.7
72		Ae	2.0	2.2	0.5
73		Bt	3.0	8.6	0.05
74		Ck	4.0	8.4	0.02
75	Dark Grey Wooded	LF	0.3	7.4	8.3
76		Ahe	0.5	1.7	21
77		Ae	1.0	1.0	1.7
78		Bt	2.0	1.9	0.07
79		Ck	4.0	1.8	0.03
80	Grey Wooded	LH	0.2	16.6	64
81		Ae	0.5	1.4	0.9
82		Bt	1.5	6.4	0.06
83		C	3.0	9.1	0.06

Sample No.	CEC (me./100g.)	pH	%N	% Total C	% In-organic C	C/N	% Free Fe ₂ O ₃	Soluble Fe (ppm.)
64	33.0	6.5	0.32	3.82	—	12	0.09	8
65	18.2	6.2	0.05	0.48	—	10	0.02	7.5
66	12.8	8.0	0.03	0.95	6.3	—	0.03	7.5
67	36.1	6.7	0.49	5.95	—	12	0.07	3
68	6.9	6.2	0.05	0.67	—	13	0.04	8
69	33.4	6.4	0.07	0.99	—	14	0.04	6.5
70	26.2	7.6	0.05	0.90	4.5	—	0.04	5.5
71	24.4	6.7	0.26	3.23	—	12	0.04	0.5
72	11.5	6.1	0.06	0.64	—	11	0.04	4
73	27.7	5.2	0.04	0.53	—	13	0.03	7.5
74	19.6	6.9	0.04	0.50	1.0	—	0.03	3.5
75	54.3	6.8	1.09	15.2	—	14	0.03	3
76	12.3	6.1	0.09	1.04	—	12	0.03	6
77	4.7	6.7	0.03	0.43	—	14	0.01	2.5
78	9.5	6.4	0.03	0.36	—	12	0.01	2
79	8.1	8.1	0.02	1.08	6.4	—	0.002	8.5
80	37.8	6.4	0.53	10.6	—	20	0.02	2.5
81	5.8	5.1	0.04	0.45	—	11	0.04	8
82	17.3	4.8	0.03	0.58	—	19	0.03	11
83	31.4	5.1	0.03	0.45	—	—	0.07	12

reducing population. NH_4^+ -acetate soluble iron and oxalate extractable free iron content has no effect on size of population.

Properties of the soil utilized in ecological studies:

The properties of soil sample G are shown in Table 17. In most cases this was well suited to these ecological studies, although it may have been too fertile to estimate the effect of organic matter addition.

Table 17. Properties of the black Chernozemic Ah soil (Sample G) used in ecological studies.

Moisture content (%)	2.2
pH	6.8
Total C (%)	12.4
Total N (%)	0.86
C/N	14
Total cation exchange capacity (me./100 g.)	67.6
Free iron (%)	0.04

Effect of temperature on the iron reducing population of soil:

- Reduction at various temperatures by pure and mixed cultures.

A psychrophile is defined to be unable to grow at 30°C . and have its optimum growth at 18°C . to 20°C .; a mesophile's optimum is between 30°C . and 37°C .; an obligate thermophile shows no growth at 37°C . and maximum growth at 65°C . or greater; and a facultative thermophile may

grow at 37°C. and grows best at 55°C. (Stanier, 1963).

Culture 92 is essentially a psychrophile (Table 18) since it has difficulty growing at 30°C. in iron broth. The only medium in which this organism grows well above 30°C. is Vitamin B12 denitrifier broth, another indication of the increased temperature tolerance given by this vitamin. Speed of reduction at 15°C. is not much slower than at room temperature, and the organism can reduce iron at 5°C. fairly rapidly.

Isolate 130 was typical of the majority of our isolates in showing both psychrophilic and mesophilic reduction. Mixed culture JB grew over a narrower range of temperature, reducing iron only at room temperature.

Organisms in soil culture IR16 (Humic Gleysol B) could reduce Fe^{+++} from 2°C. to room temperature. Soil culture IR2 (manured garden soil) contained, in addition, thermophilic reducers. Results for these soils were verified by enrichment cultures, which reduced Fe^{+++} more quickly than had the primary cultures.

- Thermophilic iron reduction.

The enriched thermophilic iron reducing culture from soil IR16 was carried on successfully at 65°C. for two further transfers, but it was ultimately lost. Further attempts to obtain cultures capable of reducing iron at 65°C. from soils IR2, G, and manure have been unsuccessful. It is thought that this organism could be more easily isolated from manure since high temperatures can develop

in piled compost.

Table 18. Iron reduction by pure and mixed cultures at various temperatures in B10 broth.

Culture	Temperature (°C.)										
	2	5	10	15	22-26	30	34	37	40	50	65
control	—	—	—	—	—	—	—	—	—	—	—
92	—	7	4	2	2	—	—	—	—	—	—
130	—	—	4		2		2	—	—	—	—
JB	—	—	—		2			—		—	—
IR16:											
primary	42	21	7		3			—		—	—
enriched	21	14	5		1			—		—	—
IR2:											
primary	42	21	7		2			2		2	4
enriched	21	14	5		1			1		1	1

Code:

- 3 strong visible reduction after 3 days
 — no reduction

- Effect of temperature on the iron reducing population of a water-logged soil

Figure 8 illustrates the MPN of iron reducers and NH_4^+ -acetate soluble $\text{Fe}^{++} + \text{Fe}^{+++}$ and Fe^{++} in waterlogged samples of soil G incubated at various temperatures for a month. Numbers are seen to increase to 15°C. and remain stable to room temperature. There is a decrease at 40°C. and a population peak at 50°C. No organisms were found in the 65°C. sample. Total soluble Fe and soluble Fe^{++} is not very well correlated with population. It should be noted, however, that only 2 to 15 days are required to complete the reduction process

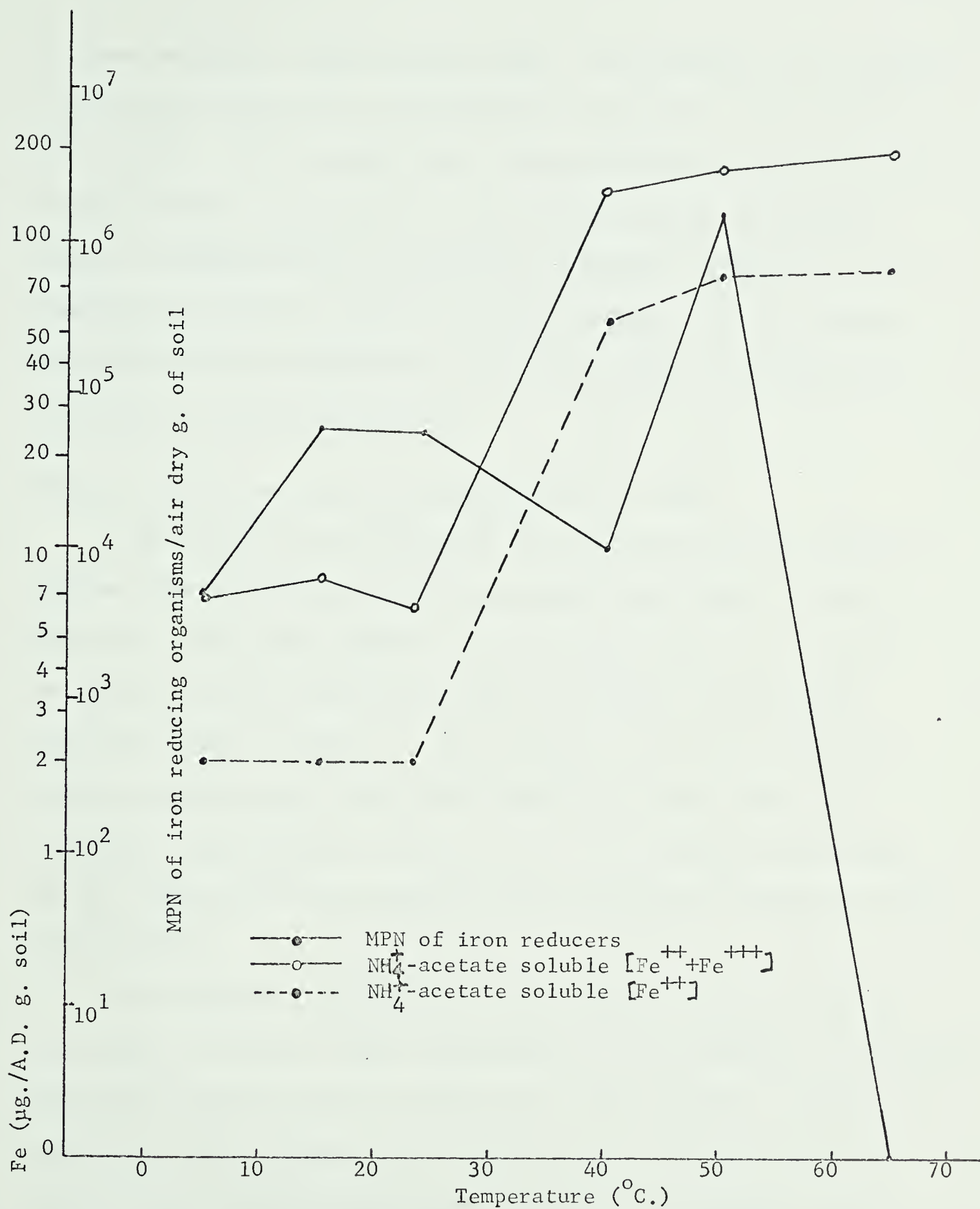


Figure 8. MPN of iron reducers, soluble $[\text{Fe}^{++}]$, and total soluble [iron] in waterlogged topsoil incubated at various temperatures for 4 weeks. Moisture content of soil = 100%.

at room temperature (Takai et al, 1963a). These samples were incubated for one month, and what is measured here is the final concentrations of iron species. For the same reason, the populations shown here probably do not represent the numbers of iron reducers present during their period of maximum activity, excepting the samples incubated at cooler temperatures where the process is slower (McKeague, 1965). Further experiments should concentrate on following reduction and population with time at various temperatures.

Effect of pH on the iron reducing population of soil:

The initial pH encountered by the microflora of soil G samples and the final pH after soil buffering action for 5 weeks is shown in Table 19. This initial drastic acidity or alkalinity in the samples of high or low pH no doubt accentuated the change in iron reducer population shown in Figure 9. There appears to be fair correlation between population and soluble Fe^{++} and total soluble iron, with all three rising sharply at pH 8.2 to 8.4. The possible relationship of Fe^{++} concentration and population with increasing pH deserves further consideration.

The increase in population of iron reducers with increased pH is a point of interest since there appears to be an analogy with sulphate and nitrate reducers, both of which also prefer high pH. An effort to demonstrate this phenomenon with saline topsoil from the edge of a slough near Vulcan, Alberta yielded relatively high numbers of iron reducers (moisture content = 40%, MPN of iron reducers = 69,000/O.D.

Table 19. Initial pH encountered by soil microflora of sample G, and final pH after 5 weeks incubation at room temperature

Sample	Reagent (20 ml.) added to 10 g. of soil G	pH of sample over time		
		$\frac{1}{2}$ hr.	1 day	5 weeks
1	0.3 N HCl	2.9	3.2	3.6
2	0.2 N HCl	3.6	4.0	4.5
3	0.1 N HCl	4.7	5.4	5.9
4	0.05 N HCl	5.6	6.1	6.6
5	0.01 N HCl	6.7	7.0	7.0
6	distilled water	7.4	7.4	7.5
7	0.01 N NaOH	8.6	8.2	7.5
8	0.05 N NaOH	10.1	9.5	8.2
9	0.1 N NaOH	11.0	10.1	8.4
10	0.2 N NaOH	12.1	11.5	8.7
11	0.3 N NaOH	12.3	11.9	9.1

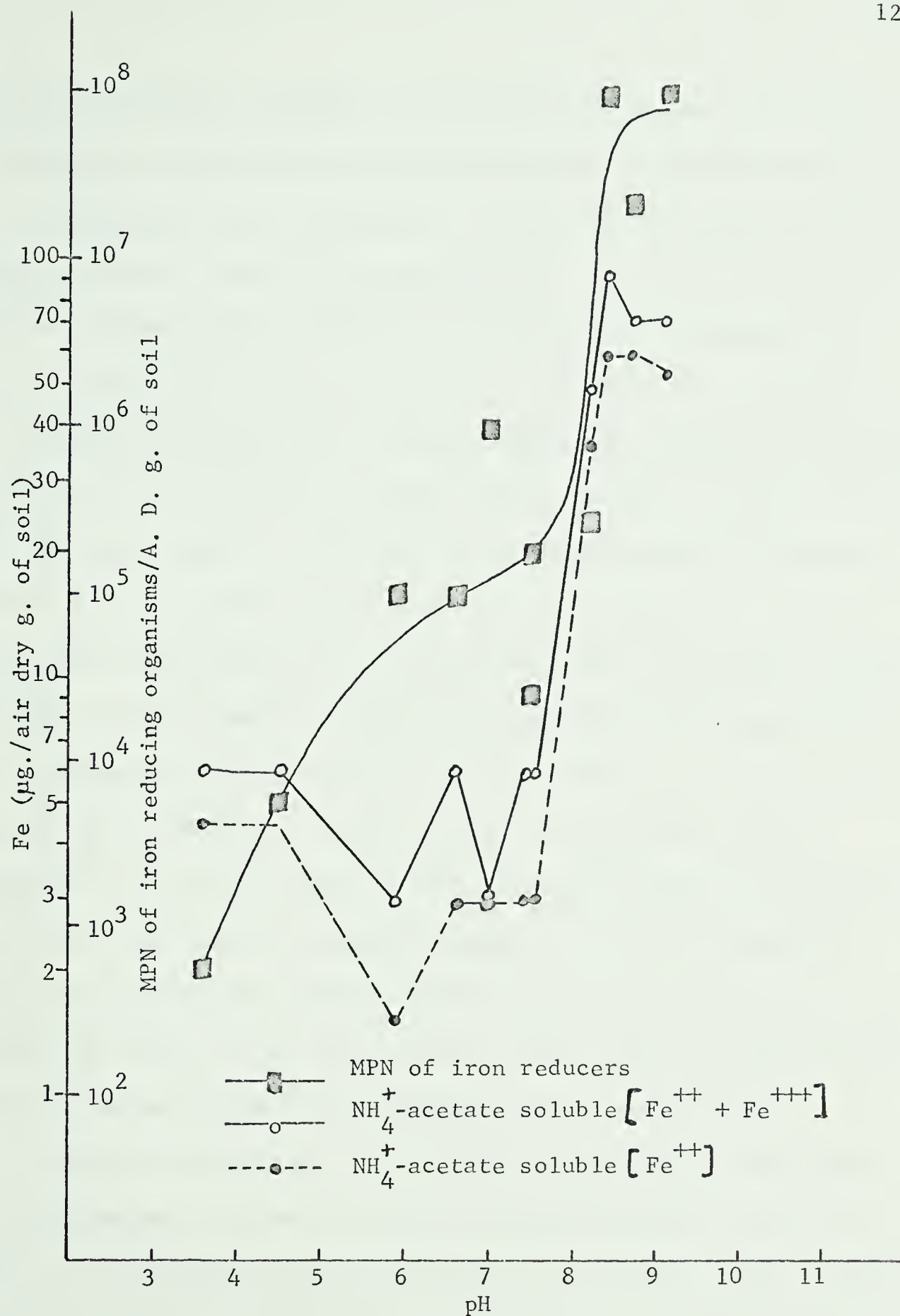


Figure 9. MPN of iron reducers, soluble $[\text{Fe}^{++}]$, and total soluble [iron] in waterlogged topsoil samples amended to various pH levels and incubated for 5 weeks. Moisture content = 200%.

g. of soil) but not remarkable, since the pH (7.6) was not as high as expected. A strongly alkaline Solonetzic soil should be examined.

Isolate 92 showed tolerance to alkalinity but not great acidity; the pH range over which it has reduced Fe^{+++} is 5.8 to 9.1, with the top limit being unknown. Sterile controls at pH 9.1 dropped to pH 8.4. The upper limit of pH tolerance is not known for isolate 92.

The pH of primary iron reducing cultures, from the soil samples at pH 3.6 to 9.1 and grown in B10 at these reactions, showed a moderation of these reactions. Those grown at pH 3.6 showed an increase of pH to 5.9 to 6.2, while the sterile controls increased to 5.0 and showed only slight reduction. At the other end of the scale, sterile controls at pH 8.7 and 9.1 decreased to pH 8.0 and 8.4 respectively. The cultures grown at pH 9.1 for a week decreased pH to 7.6 to 8.4; those at pH 8.7 decrease to pH 7.3 to 8.1; and those at pH 8.4 dropped to 7.7 to 8.3. Those at neutral or near-neutral pH remained essentially the same or increased somewhat. Cultures incubated at pH 8.4 generally showed a white ferrous phosphate precipitate. Those cultures at pH 8.7 or 9.1 often produced dark green or black precipitates, which are thought to be $\text{Fe}(\text{OH})_2$ and FeS respectively.

General description of the colonial morphology on B10 plate of the organisms obtained from these primary cultures is given in Table 20. When these primary cultures were plated for single colonies on both B10 plates at neutral pH and on B10 plates at the pH of the primary culture, there was essentially no difference, indicating that all iron reducing organisms could grow at neutral pH. It was thought

that isolates 1-8 or 92 might be isolated with this procedure but no agar depressors were obtained. However, alkaline B10 broth would be an excellent medium for isolation of good iron reducers.

Table 20. Colonies obtained from primary soil cultures (in B10 at various pH levels) plated on B10-agar amended to the pH of the culture.

pH of primary culture	pH of B10 plate	Description of Colonies
3.6	4.0 (semi-solid)	Primary cultures showed fungal pellicles but growth in semi-solid B10 was bacterial.
4.5 5.8 6.6 7.1 7.5	4.8 6.1 6.6 7.0 7.5	<u>B. circulans</u> type of culture (pebbled white); and yellow-brown cultures which are generally poor reducers, non-reducing citrate fermenters, or good reducers like isolate 8-2.
8.2 8.4 8.7 9.1	8.0 8.3 8.7 9.0	Small white cultures (2 mm.) with entire or rough edge like isolate 6 in the majority and usually in pure culture at higher pH. Also bright yellow to pale yellow like isolates 130 or 633.

Effect of addition of Fe_2O_3 :

Table 21 illustrates that there was actually a decrease in the iron reducing population and little increase in soluble iron species with the addition of Fe_2O_3 to soil G.

Table 21. Effect on MPN of iron reducers, total soluble [iron], and soluble $[\text{Fe}^{++}]$ with the addition of Fe_2O_3 to waterlogged soil. Moisture content = 100%. Incubation period = 5 weeks.

Wt. of Fe_2O_3 added to 50 g. A.D. soil (g.)	MPN of iron reducers per A.D. g. soil	NH_4^+ -acetate soluble iron	
		$[\text{Fe}^{++} + \text{Fe}^{+++}]$ ($\mu\text{g.}/\text{A.D. g. soil}$)	Fe^{++} ($\mu\text{g.}/\text{A.D. g. soil}$)
0.0	480,000	8	2
2.0	66,000	11	1
4.0	46,000	12	3

Effect of addition of organic matter:

When a C source in the form of oat straw was added to soil G, an increase in soluble iron species and an apparent increase in the iron reducer population was observed (Table 22). Addition of C+N in alfalfa intensified this process. However, the increase in population is not as marked when the population of the control of Table 22 is compared with the control of Table 21. Both controls were incubated under essentially the same conditions, and although in comparison with other controls that of Table 21 is abnormally high, and that of Table 22 low, it is apparent that a soil containing less organic matter would have been more suitable for the experiment.

Table 22. Effect on MPN of iron reducers, total soluble [iron], and soluble $[\text{Fe}^{++}]$ with the addition of organic matter to waterlogged soil. Moisture content = 100%.
Incubation period = 5 weeks.

Amendment to 100 g. A.D. soil	MPN of iron reducers per A.D. g. soil	NH_4^+ -acetate soluble iron	
		$[\text{Fe}^{++} + \text{Fe}^{+++}]$ ($\mu\text{g.}/\text{A.D. g. soil}$)	$[\text{Fe}^{++}]$ ($\mu\text{g.}/\text{A.D. g. soil}$)
Nil	8,200	7	2
2% oat straw (C)	260,000	144	52
2% alfalfa (C+N)	660,000	176	64

Effect of moisture and anaerobiosis:

Reasonably direct relationship is seen in Figure 10 between the increase in numbers and in the concentration of iron species with increased moisture, which is a reflection of increased anaerobiosis.

Comments on laboratory experiments with the ecology of iron reducers:

Iron reducing populations have been shown to increase with added organic matter, moisture, and pH. They tend to increase with temperature up to 50°C., but not with addition of Fe₂O₃. In nearly all cases total soluble iron and soluble Fe⁺⁺ are related to population, and it is probable that although the iron reducers may not be directly responsible for the solubilization of iron, they are responsible for the high levels of Fe⁺⁺ found; particularly, for example, at the alkaline pH levels, where the predicted result by chemical consideration alone would be low Fe⁺⁺ levels (Starkey and Halvorson, 1927).

These ecological studies are preliminary and have been performed after the iron reducing processes in these samples were complete. Further studies should no doubt follow the process over time in more detailed fashion.

In retrospect, it appears that one crucial experiment is missing in this thesis. Several workers have followed the iron reduction procedure over time, linking it with carbohydrate utilization (Kumura et al, 1963); Takai et al, 1963b), decrease of redox potential or increase in pH (McKeague, 1965; Takai et al, 1963ab),

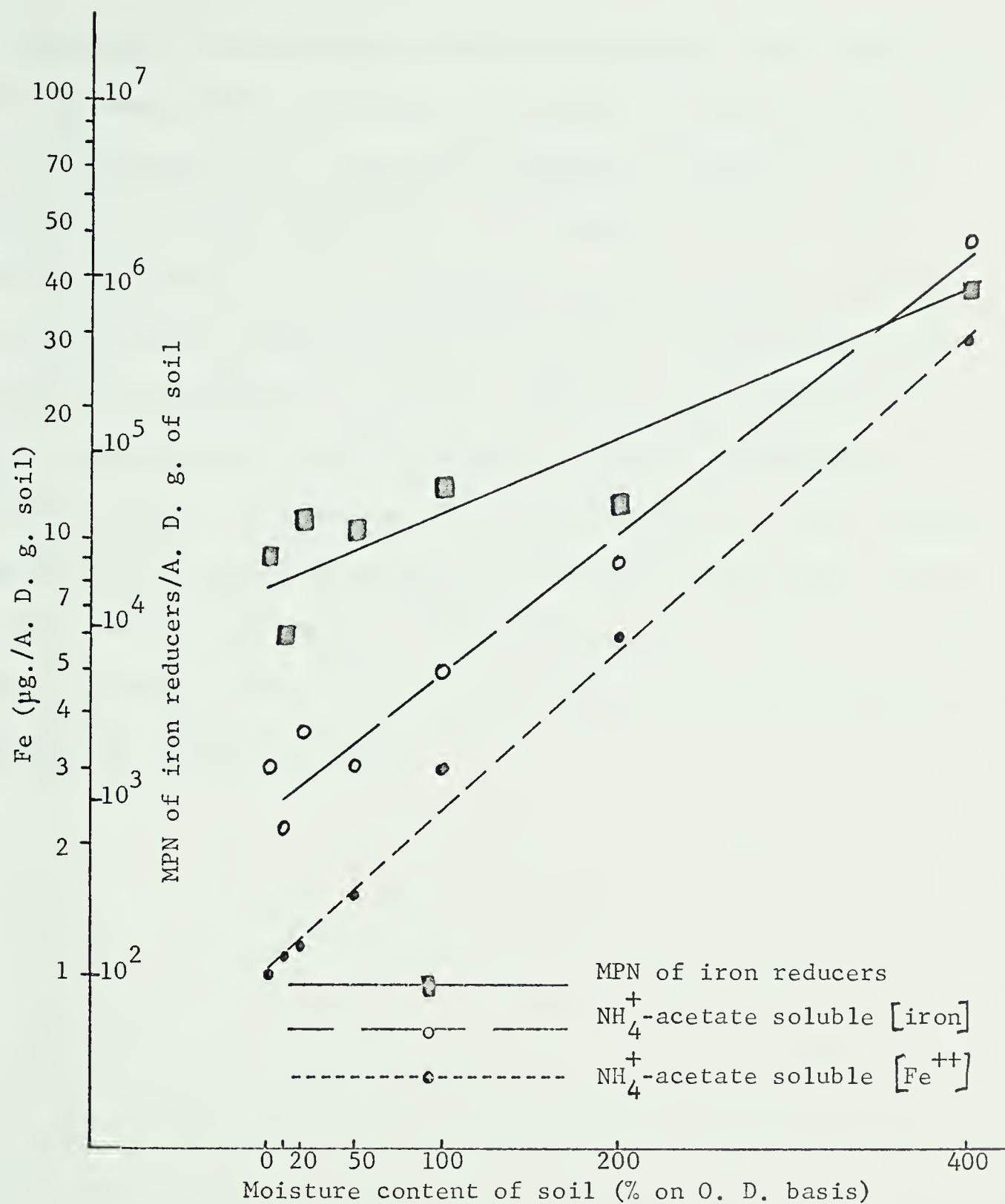


Figure 10. MPN of iron reducers, soluble [Fe^{++}], and total soluble [iron] in topsoil samples incubated at various moisture levels for 5 weeks.

or following it over a temperature range (McKeague, 1965). Takai et al (1963ab) have showed the sequence of microbial reductions which occur in a waterlogged soil. The missing experiment referred to is the measurement of the relationship of iron reduction, MPN of iron reducers, increase in pH, and decrease of redox potential over time. A more ambitious undertaking would follow MPN of denitrifiers, iron reducers, and sulphate and sulphite reducers with disappearance of NO_3^- , and reduction of Fe^{+++} and $\text{SO}_4^{=}$, with other measurements, to provide a further elucidation of the relationship of the waterlogging processes with direct reference to the microbial populations involved. A great deal of overlapping of MPN curves could be expected due to the ability of many of these organisms to utilize more than one inorganic compound as electron acceptor.

Solubilization and Reduction of Iron Compounds by Plant Extracts and Iron Reducing Cultures

Comparison of nature of reduction of Fe^{+++} by aspen poplar leaf extracts and isolate 92:

Addition of the 10^{-1} dilution of aspen leaf extract to B10 caused an immediate blackening of the media. There was no effect when extract was added to control A3, so the reaction was with the iron. Addition of the 10^{-2} dilution resulted in a dark green color; the 10^{-3} dilution, a light green; and increased dilutions, no visible reaction even after 2 days. Addition of 10^{-1} dilution of leaf extract to a well-reduced culture of isolate 92 also caused a blackening of the medium, indicating that the plant materials also react with Fe^{++} as well as Fe^{+++} . Spot tests of B10-leaf extract with 2% o-phenanthroline gave no positive reaction for Fe^{++} due to the strong chelate formed by the leaf extract components (Bloomfield, 1951).

For those B10-leaf extract cultures inoculated with 92, no gas was produced, but a strongly positive spot test for Fe^{++} was recorded except for the tubes amended with the 10^{-1} dilution of the extract. Whether isolate 92 reduced Fe^{+++} in the organic combination is unknown.

Comparison of methods for extracting iron from organic combination, and extent of reduction of soluble Fe^{+++} by aspen poplar leaf extract:

Comparison of methods for extraction of iron from its combination with leaf extracts revealed that total recovery could be obtained with

the pH 4.8, 1 N, NH_4^+ -acetate extraction method (Olson, 1965). In fact, this extractant was not required, for total recovery was obtained when the B10-leaf extract was mixed with the pH 3.5 Na-acetate-acetic acid buffer used in the regular procedure for colorimetric determination of iron (Table 23). However, in the case of the 3% AlCl_3 extracting solution (Ignatieff, 1941), while there was no difference between standard curves for Fe^{++} in acetate or acetate + AlCl_3 (Appendix III), there was interference with total recovery of iron from organic combination. For the solubilization experiments which follow, the pH 4.8 NH_4^+ -acetate extraction was used because insoluble precipitates also were present.

It is evident that the plant extracts had considerable capacity to reduce Fe^{+++} . However, the extract was obtained by heating and is in much higher concentrations (about 1.5%) than would be found in soil.

Table 23. Recovery of iron from combination with aspen poplar leaf extracts with different extraction methods; and extent of reduction of soluble Fe^{+++} in 1.5% leaf extract solution.

Solution Tested	Extracting Solution	Total Fe ($\mu\text{g.}/\text{ml.}$)	Fe^{++} ($\mu\text{g.}/\text{ml.}$)
B10	Nil	560	160
B10-leaf extract	Nil	560	460
B10-leaf extract	1 N, pH 4.8, NH_4^+ -acetate	560	480
B10-leaf extract	3% AlCl_3	480	350

Solubilization and reduction of insoluble iron compounds:

Table 24 illustrates that bromegrass had little capacity to solubilize and reduce Fe_2O_3 compared to a soil culture. Reduction of Fe^{+++} is increased when soil microbes are fed grass as a substrate. Individual isolates on the average were just as effective as mixed cultures, indicating that they do not require other organisms to perform the solubilization step for them. There was no correlation between the quantity of anaerobic growth in A3 medium and the solubilization of iron, indicating that the solubilization process, rather than depending on solution by organic metabolites as Daragan (1966) suggests, is probably directly due to an equilibrium reaction set up by the reduction process itself. Otherwise isolates 6 or 92, which grow poorly in A3 under anaerobiosis, would not have been able to perform as well as they did.

Table 24. Solubilization and reduction of Fe_2O_3 by pure iron reducing cultures, soil cultures, and bromegrass. Weight of Fe_2O_3 = 0.1 g. Incubation period = 10 days.

Culture	NH_4^+ -acetate soluble iron	
	Fe^{++} (ug.)	Total Fe (ug.)
Sterile Fe_2O_3 in A3 medium	0	25
Soil + Fe_2O_3	40	120
Sterile grass + Fe_2O_3	25	25
Soil + grass + Fe_2O_3 } corrected for Fe in soil inoculation	130	150
1-3	40	90
1-4	105	160
1-6-2	50	120
1-8	50	95
1-10	50	95
1-12-2	40	95
3	10	15
6	295	440
7-2	270	345
8-2	25	80
12-4	190	190
14-4	30	50
19	70	130
22	200	230
92	105	145
130	215	130
627	30	48
629-3	105	305
633	215	320
634	95	70
635	105	150
636	130	280
I2	55	105
<u>B. circulans</u>	30	160
<u>B. mycoides</u>	25	40
<u>B. subtilus</u>	10	10

Only isolate 92 was capable of solubilizing a small quantity of the hematitic rock and outperformed the other isolates in solubilizing and reducing the two iron ore samples (Table 25).

Table 25. Solubilization and reduction of hematitic rock and iron ores by iron reducing isolates. Weight of insoluble material = 0.1 g. Incubation period = 10 days.

Iron Source	Culture	NH ₄ ⁺ -acetate soluble iron	
		Fe ⁺⁺ (μg.)	Total Fe (μg.)
Hematitic rock	Sterile	0	70
	92	70	90
	130	70	70
	14-4	40	70
Iron ore:CH5	Sterile	20	70
	92	180	740
	130	40	90
	14-4	0	70
Iron ore:CH12	Sterile	20	70
	92	40	110
	130	20	70
	14-4	0	20

Gleization as a Microbial Process

Table 26 illustrates reduction and solubilization of iron in a Bm horizon of an Orthic Black Chernozem amended with A3 medium as substrate. Again, action of pure iron reducing cultures are comparable to the action of a mixed flora, which is contrary to the hypothesis of Bromfield (1954b), who felt that a mixed culture would be more effective in solublizing iron.

Table 26. Gleying of an Orthic Black Chernozem Bm sample amended with A3 broth by pure and mixed iron reducing cultures. Weight of soil = 1.0 g. Incubation period = 10 days.

Culture	NH ₄ ⁺ -acetate soluble iron		Visual evidence of gleying
	Fe ⁺⁺ (ug.)	Total Fe (ug.)	
Sterile	20	40	none
92	150	380	strong
14-4	40	90	none
Lawn soil (corrected for Fe in soil inoculum)	70	240	fair

Table 27 shows that sterile brome grass has by no means the capacity to cause gleying that a microbial culture has. Addition of brome grass to a mixed microbial culture intensifies the gleying process because it is utilized as substrate.

Table 27. Comparison of effect of brome grass and native microflora on gleying in an Orthic Black Chernozem Bm sample. Weight of soil = 1.0 g. Incubation period = 10 days.

Culture	NH ₄ ⁺ -acetate soluble iron		Visual evidence of gleying
	Fe ⁺⁺ (ug.)	Total Fe (ug.)	
Sterile soil	0	20	none
Sterile (soil + 0.1 g. grass)	0	40	none
Non-sterile soil	110	110	slight
Non-sterile (soil + 0.1 g. grass)	330	510	strong

Selected examples of the Bm soil cultures set up to provide visual proof of the microbial nature of gleying are shown in Plates 4, 5, 6, and 7. Only autoclaved controls are shown, since the radiation procedure turned the glass of γ -radiated tubes brown. Isolate 92 (not shown) showed good gleying 2 weeks after inoculation into autoclaved A3 plus soil. It was less successful in the radiated soil. After 3 weeks the non-sterile soil sample amended with A3 (Plate 7) was strongly gleyed while the sterile control remained brown. Strong gleying of the non-sterile aspen poplar leaf amended soils occurred in 4 weeks, while little occurred in the sterile tubes,



Plate 4. Microbial gleying in a sample of Bm horizon of an Orthic Black Chernozem (Pincher Creek) after 3 months of water-logged conditions. Magnification: 2.5 X.

Left: Non-sterile sample. Right: Steam-sterilized sample. There is approximately a one inch depth of soil covered by a three inch depth of water. The container is a screw-capped vial. This stage of gleying was reached after 6 weeks of incubation.



Plate 5. Microbial gleying in a 5 gram sample of chermozemic Bm soil amended with 0.1 gram of ground aspen poplar leaves.

Magnification: 2.5 X.

Left: Non-sterile sample. Right: Steam-sterilized sample. There is a brown zone of decomposed leaf matter in the left tube. The ground leaf fragments can be seen, undecomposed, on the soil-water interface of the right tube. This stage was reached after 6 weeks incubation.



Plate 6. Microbial gleying in a 5 gram sample of chernozemic Bm soil amended with 1 gram of ground aspen poplar leaves.

Magnification: 1.5 X.

Left: Steam-sterilized sample. Right: Non-sterile sample. Some reoxidation, due to leakage of air, has occurred in the non-sterile sample and mottles can be seen near the soil-organic layer interface. A duplicate non-sterile sample showing gleying did not develop leakage or show mottling. This stage was reached within 6 weeks incubation.



Plate 7. Microbial gleying in a sample of chernozemic Bm soil submerged in a nutritionally rich iron-free growth medium.
Magnification: 2.5 X.

Left: Non-sterile sample. Right: Steam-sterilized sample. The medium used was A3, control medium for B10. This stage was reached after three weeks incubation. Reduction of soil iron and sulphate in the medium has resulted in a visible precipitation of ferrous sulphide in the non-sterile sample.

where the leaves remained undecomposed (Plates 5 and 6). Non-sterile unamended soils were gleyed to the extent shown in Plate 4 within 6 weeks. The difference between the sterile and non-sterile unamended soils is not strong but is quite apparent. These plates are proof of the microbial nature of the gleization process.

Investigation of Siderite Production by Iron Reducing Cultures

As early as 1923, Tache found FeCO_3 accumulation in anaerobic portions of bog, and the question remains as to whether siderite present with organic matter is of microbial origin. B10 and B19 cultures, because of their high phosphate content, produced only vivianite (ferrous phosphate) precipitates, B7 cultures were utilized since, although iron reducers do not produce CO_2 , other organisms producing enough of this gas in mixed culture could cause precipitation of FeCO_3 under anaerobic conditions. However, even in B7 vivianite precipitates are formed. Current work is being carried out to devise a medium suitable for the production of siderite.

Iron Reduction and Iron Corrosion

Starkey (1945) noted that in corrosion of iron under anaerobic conditions, the process begins with a polarization of the metal, with an accumulation of hydrogen. Sulfate reducing bacteria depolarize the system by removing hydrogen and oxidize it as sulfate is reduced. The ionized Fe then reacts with OH^- and $\text{S}^{=}$ to form corrosion products. Corrosion by nitrate has been shown in our laboratory. The following corrosion experiments were undertaken to determine whether

reduction of iron could also serve to depolarize and corrode metal.

Five organisms which corrode iron during nitrate reduction, including isolate '1' (Aerobacter aerogenes) and four facultative aerobes, were tested for iron reducing capability in B10. '51' proved to be a good reducer, '1' a poor reducer, and '28', '60', and '25' non-reducers.

In a reverse procedure, iron reducers were tested as iron corrodors. Table 28 illustrates that several iron reducers are iron corrodors through their ability to reduce nitrate in the iron corrosion medium, being as effective as Cook's best corroding organism. Isolate 130, which does not reduce nitrate, had no corroding effect. KNO_2 , however, also corroded iron, which indicated that NO_2^- was responsible rather than microbial action by depolarization.

There was no corrosion when iron corrodors and iron reducers were tested in B10 broth plus iron wire. It appears that the iron corrosion capability of iron reducers is through their ability to reduce nitrate.

Table 28. Corrosion of iron with nitrate reduction by iron reducing cultures.

Culture	Weight of Wire Before Incubation (mg.)	Weight of Wire After Incubation (mg.)	Weight Lost (mg.)	NO ₃ ⁻ → NO ₂ ⁻	NO ₃ ⁻ → gas
'1'	35	26	9	+	-
92	37	26	11	+	-
130	41	41	0	-	-
1-6-2	34	30	4	+	-
627	40	29	11	+	+
control	39	38	1	-	-
control	38	38	0	-	-
control + KNO ₂	29	23	6		
control + KNO ₂	30	22	8		

Further Isolations and Miscellaneous Experiments

Reduction of Iron by Fungi

Lieske (1921) observed the reduction of iron by molds. His postulation that this could be an enzymatic process was discounted by Starkey and Halvorson (1927), who considered this to be due to the fungal pellicle excluding oxygen from the medium and creating reducing conditions through anaerobiosis.

Good growth with thick pellicles was usually obtained with fungal isolates in B7 and B10 broths. Occasionally fair reduction, by visual estimation and by spot test with 2% o-phenanthroline, was obtained with a few cultures with no hint of bacterial contamination. However, no culture was predictable in its ability to reduce Fe^{+++} . The mechanism of reduction is not yet known, but it is certainly not due to anaerobic effects. Table 29 illustrates iron reduction by ten day fungal cultures in tubes of B10 broth.

Table 29. Reduction of Fe^{+++} by fungal cultures in B10 broth.
Incubation period = 10 days. Total iron = 640 ug./ml.

Culture	Classification	Fe^{++} (ug./ml.)
Control		160
F81	?	120
F107	?	120
F110A	<u>Chrysosporium</u>	220
F110B	<u>Chrysosporium</u>	130
F112A	?	160
F112B	<u>Penicillium</u>	200
F112C	<u>Penicillium</u>	300
F118A	?	300
F118B	<u>Penicillium</u>	350
F133	?	220

Relation of Citrate Fermentation and Iron Reduction

None of the iron reducing isolates produce gas in B7 (ferric ammonium citrate) medium. Previously held cultures which fermented citrate and reduced iron ultimately lost their iron reducing properties over time in B7 broth, but not ability to ferment citrate. Any citrate fermenting, iron reducing culture which was examined for colonial morphology on B10 plate turned out to be a mixed culture. Recent isolations by soil enrichment with B7 broth have yielded citrate fermenters and iron reducers, but no organisms which accomplish

both processes. The author is not prepared to state definitely that these organisms do not exist, but the indication is that the process of citrate fermentation and CO₂ production is parallel but not directly linked to the process of iron reduction.

Notes on Various Isolations and Habitats of Iron Reducing Bacteria

No actinomycete has yet been found capable of reducing Fe⁺⁺⁺. Efforts to obtain facultatively aerobic non-sporeforming bacteria, such as the Pseudomonas of Duda and Kalakutskiy (1961) and Daragan (1965) have been unsuccessful. All of our isolates appear to be Bacillus. Spore formation by these Bacillus is often difficult to demonstrate, and it could be possible that the "Pseudomonas" isolated by these authors are really Bacillus. No work has yet been done with Clostridium.

Attempts to isolate a 1-8 or 92-like organism selectively from soil with lactate or sulphite media were unsuccessful. Other organisms were found to utilize lactate as an energy source while reducing iron, or to reduce sulphite.

Iron reducing organisms have been found in all soils and peats examined to date. They have also been found in hot springs (Banff) in association with sulphate reducers. Another habitat appears to be the rumen of cows, although only mixed cultures from this source have been shown to date to reduce Fe⁺⁺⁺.

VI. SUMMARY AND CONCLUSIONS

Facultatively aerobic, heterotrophic organisms capable of reducing Fe^{+++} have been isolated from soil. Experiments were undertaken to classify and characterize these isolates, determine the mechanism by which they reduced iron, and to illustrate various aspects of their ecology and function in soil.

The following conclusions have been drawn:

1) All iron reducing bacteria isolated to date are Bacillus. Most have been identified as belonging to Group 2 (Smith et al, 1952) of this genus. Several resemble Bacillus circulans, but others are unique. If further research bears out the fact that new species have been isolated, Bacillus ferrireductans is one name proposed for this group of organisms.

2) Isolates 1-8 and 92 are unique in reducing NO_3^- to NO_2^- , Fe^{+++} to Fe^{++} , and $\text{SO}_3^{=}$ to $\text{S}^{=}$. The reduction of $\text{SO}_3^{=}$ has not been recorded for any other aerobic sporeformer to date.

3) As a group, these organisms do not ferment citrate, succinate, acetate, glucose, sucrose, lactate, or pyruvate to produce CO_2 , or ammonify strongly. These processes during soil waterlogging are parallel, but not directly linked, to the iron reducing process.

4) Fe^{+++} is utilized by organisms as a terminal electron acceptor in lieu of oxygen under anaerobiosis. The process is growth-bound, and sensitive to azide, cyanide, and, in a few cases, carbon monoxide. The cytochrome system is thought to be involved, and the

process analogous to respiratory nitrate and sulphate reductions.

5) An "iron reductase", similar to nitrate reductase, is postulated to exist in isolates 1-8 and 92. It is a constitutive enzyme.

6) Incidental reduction of soil iron is not as important as microbial respiratory reduction of iron.

7) Like the denitrifying population of soils, the population of iron reducing organisms is benefitted by increased organic matter, increased moisture, anaerobiosis, and high pH.

8) The soil iron reducing population can function from 2° C. to at least 50° C. Thermophiles reducing iron at 65° C. have been detected but not isolated.

9) Iron reducing isolates have shown ability to solubilize and reduce insoluble iron compounds. In this respect they are superior to plant extracts. Rather than organic acids performing the solubilization step, it appears that the iron reducing process itself sets up an equilibrium reaction to solubilize iron.

10) Although gleization can be caused by strictly chemical processes, the major cause of this process in waterlogged soils is the activity of iron reducing bacteria.

11) Iron reducers can cause corrosion of iron through their ability to reduce nitrate, but not by the iron reduction process.

12) Some fungi can reduce Fe^{+++} , but the mechanism is unknown.

This thesis has provided foundation for several future investigations. A full-scale biochemical study of the individual reactions

occurring in the cell as iron is reduced is now in order. Isolate 92 would be a suitable organism for this study. Further classification work is required for the isolates now in stock. Experiments to further elucidate the succession of reactions in a waterlogged soil in relation to population dynamics of the organisms involved would be another interesting study. Further isolations of iron reducers from soil, particularly a search for aerobic non-sporeformers, could be carried out; and a method for isolating organisms 92 and 1-8 would be desirable. The importance and nature of fungal reduction could be investigated. Finally, a study of the analogy between NO_3^- , Fe^{+++} , and $\text{SO}_3^{=}$ reductions in isolate 92 would provide an interesting research project.

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APPENDIX I. MEDIA

B: $(\text{NH}_4)_2\text{SO}_4$0.5 g./l. K_2HPO_40.5 " MgSO_40.5 " CaCl_20.2 "

Ferric ammonium citrate.....10.0 "

Adjust pH to 7.0 with 1 N NaOH.

B3:

B broth + 5.0 g./l. yeast extract

B7 routine iron broth: K_2HPO_40.8 g./l. KH_2PO_40.2 " MgSO_40.2 " NaCl0.2 " MnSO_40.002 " NaMoO_40.002 "

Yeast extract.....5.0 "

Peptone.....5.0 "

Ferric ammonium citrate.....10.0 " (approx. 1.75 g. Fe/l.)

 CaSO_4 (sat'd sol'n).....10 ml.

Adjust pH to 7.0 with 1 N NaOH.

B7 Plate:

B7 broth + 20 g./l. agar.

A1 control medium for B7 broth:

B7 minus ferric ammonium citrate. Add 6.0 g./l. Na-citrate and, to satisfy micronutrient requirements, 0.002 g./l. FeSO_4 .

B10 routine iron broth:

As in B7 but substitute 4.7 g./l. FePO_4 for ferric ammonium citrate.

B10 plate:

B10 broth + 20 g./l. agar.

A3 control broth for B10:

B10 minus FePO_4 . Increase K_2HPO_4 concentration to 6.4 g./l. and add 0.002 g./l. FeSO_4 .

B19 base medium:

K_2HPO_4 0.8 g./l.

KH_2PO_4 0.2 "

MgSO_4 0.2 "

NaCl 0.2 "

MnSO_4 0.002 "

Na_2MoO_4 0.002 "

$(\text{NH}_4)_2\text{SO}_4$ 1.0 "

FePO_4 4.7 "

Yeast extract 0.1 "

CaSO_4 (sat'd sol'n) 10 ml./l.

Adjust pH to 7.0 with 1 N NaOH. Add sterile substrate aseptically.

B19 base for resting cell medium:

As for B19 above but omit $(\text{NH}_4)_2\text{SO}_4$. Add sterile substrate.

A19 control for B19 base for resting cell medium:

As in B19 resting cell base but omit FePO_4 , increase K_2HPO_4 concentration to 5.5 g./l. and add 0.002 g./l. FeSO_4 .

B20 resting cell medium:

As in B19 resting cell medium but decrease FePO_4 concentration to 2.8 g./l. (approx. 400 ppm. Fe in medium) and add 10.0 g./l. Na-citrate. Adjust pH after autoclaving to 7.8 with sterile NaOH. The sterile substrate added is 50 ml. of 10% Na-lactate (approx. 250 ppm. lactate in medium).

A20 control for B20 resting cell medium:

As in B20 but increase K_2HPO_4 to 5.5 g./l. and add 0.002 g./l. FeSO_4 .

Vitamin B12 broth for denitrifiers:

Tryptone.....3.0 g./l.
 Yeast extract.....1.0 "
 KNO_32.0 "
 NaCl8.5 "
 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$1.47 "
 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.1% sol'n).....7.0 ml./l.
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1% sol'n).....15.0 ml./l.
 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.1% sol'n).....1.0 ml./l.
 Tris (0.64 M).....39.0 ml./l.
 Vitamin B12 (18 /ml. sol'n).....2.0 ml./l.
 pH adjusted to 7.2-7.4 with 1 N NaOH.

Vitamin B12m control broth:

Substitute 2.0 g. $(\text{NH}_4)_2\text{SO}_4$ for KNO_3 in Vitamin B12 broth.

Conc. Tris for Vitamin B12 broth:

38.59 g. Tris (THAM)

20 ml. conc. HCl.

Distilled H_2O to 500 ml.

Adjust pH to 6.8.

Plate Count agar:

Plate count.....23.5 g./l.

Heat to dissolve before sterilizing.

Butlin's medium for sulphate reducers:

K_2HPO_40.5 g./l.

NH_4Cl1.0 "

Na_2SO_42.0 "

$CaCl_2 \cdot 6H_2O$0.1 "

$MgSO_4 \cdot 7H_2O$1.0 "

Na-lactate.....2.5 "

Yeast extract....1.0 "

$FeSO_4$0.002 "

Adjust pH to 7.5. A clean iron nail is placed in the bottom of each test tube of the medium.

S1 medium for sulphate reducers:

Use B10 medium with 1.7 g./l. Na_2SO_4 substituted for $FePO_4$. pH is adjusted to 7.5. A clean iron nail is placed in the bottom of each test tube of the medium.

S2 medium for sulphite reducers:

As in S1 but substitute 1.7 g./l. Na_2SO_3 for Na_2SO_4 .

Iron Corrosion Medium:

1 part 0.64 M Tris to 3 parts water

0.5% tryptone

0.1% KNO_3

Use clean sterile nail, wire, steel wool or other iron material to demonstrate corrosion.

APPENDIX II. STANDARD CURVES FOR IRON

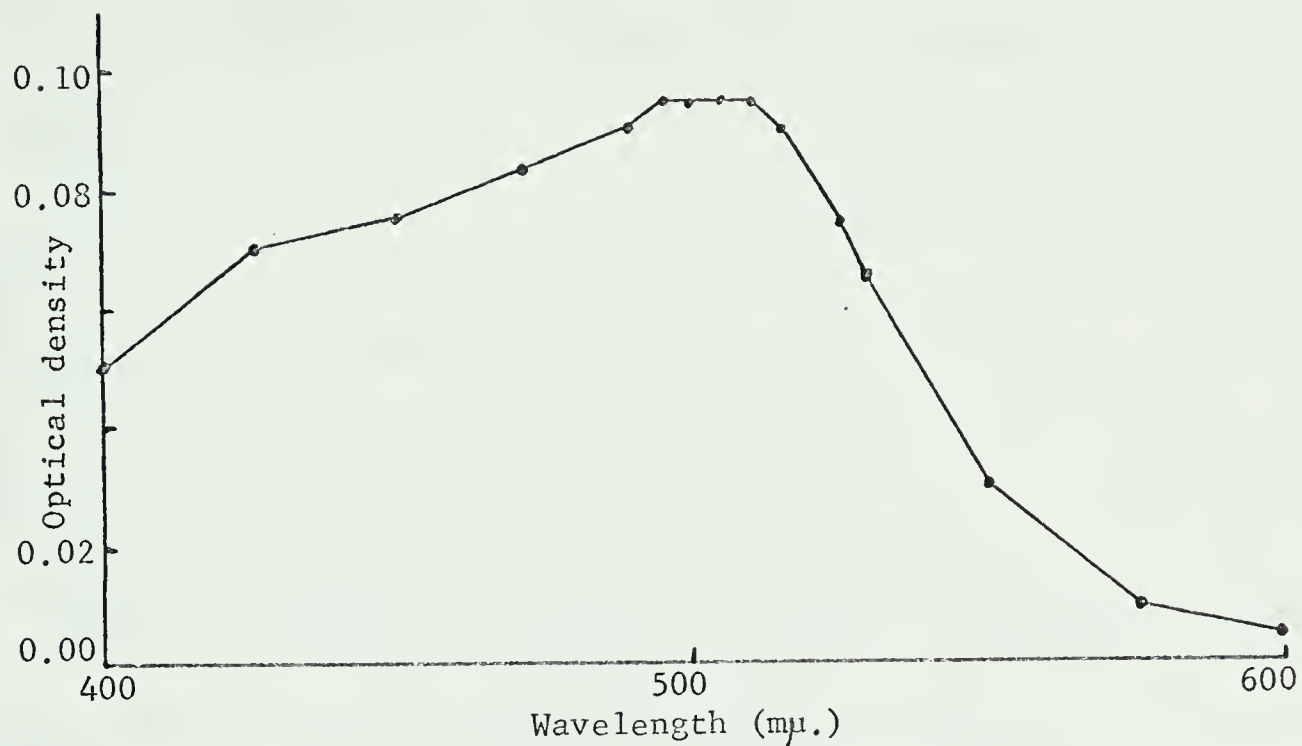


Figure 1(A2). Absorption spectra of a 1 ppm. Fe⁺⁺-o-phenanthroline solution. (Bausch and Lomb Spectronic 20.)

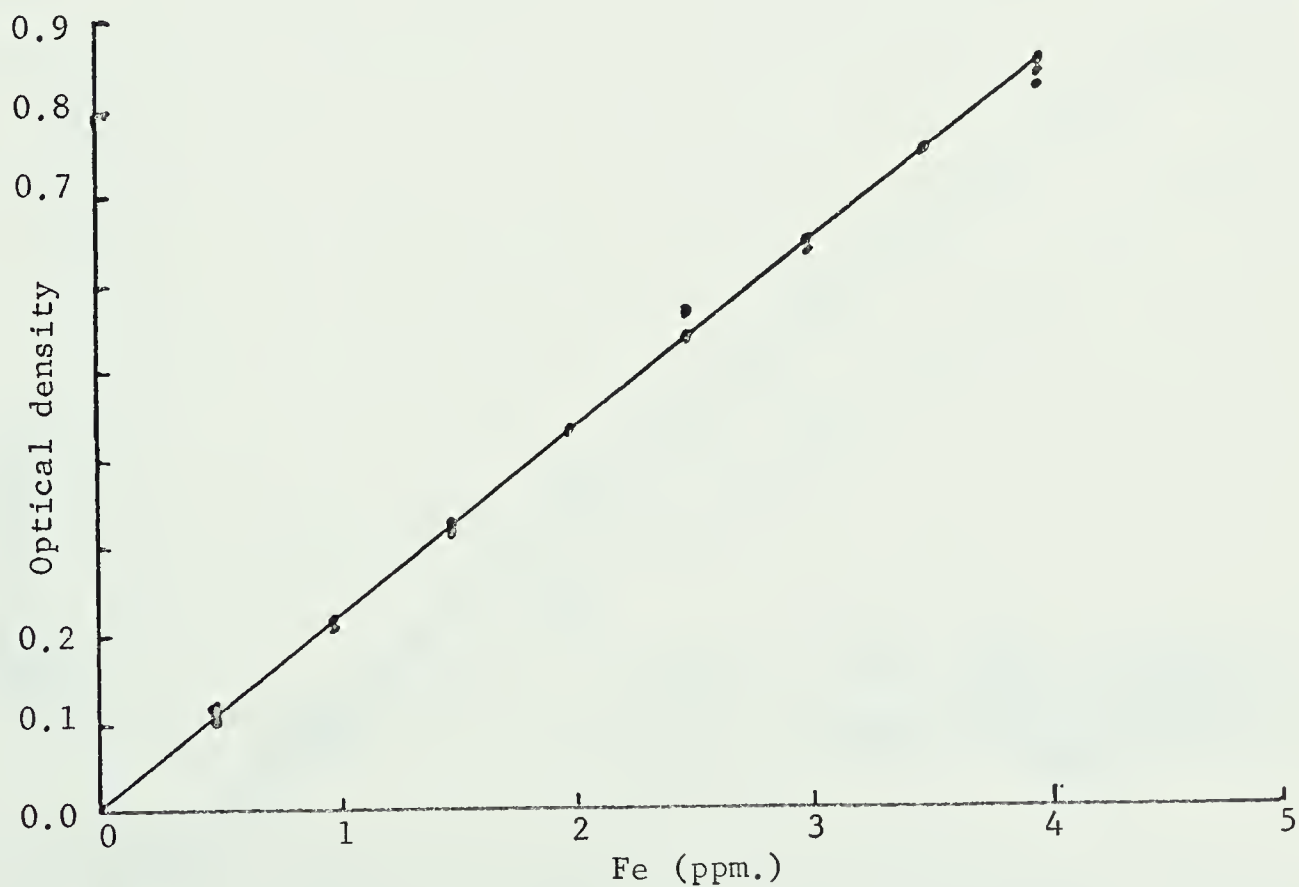


Figure 2(A2). Effect of Al, A3, A19, A20 media; and 1 N, pH 4.8 NH₄⁺ acetate and 3% AlCl₃ extracting solutions on the standard curve for Fe. (λ = 505 mμ.)

APPENDIX III. STANDARD CURVES FOR LACTATE

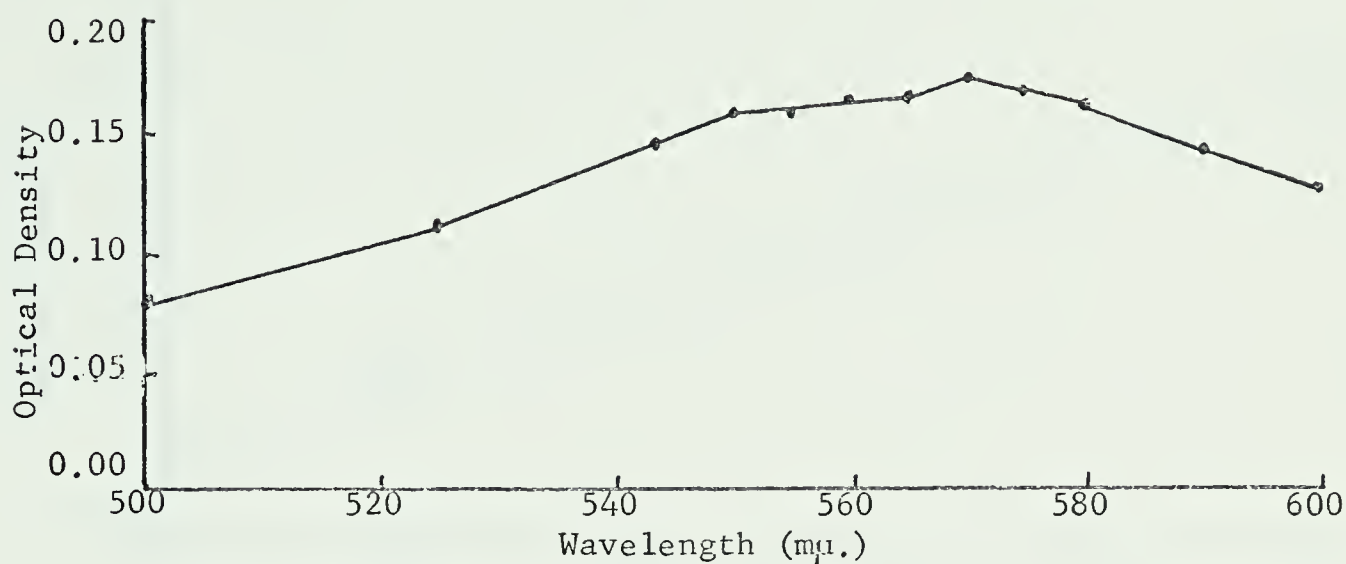


Figure 1(A3). Absorption spectra of a 1 ppm. p-OH-diphenyl-lactate solution. (Bausch and Lomb Spectronic 20.)

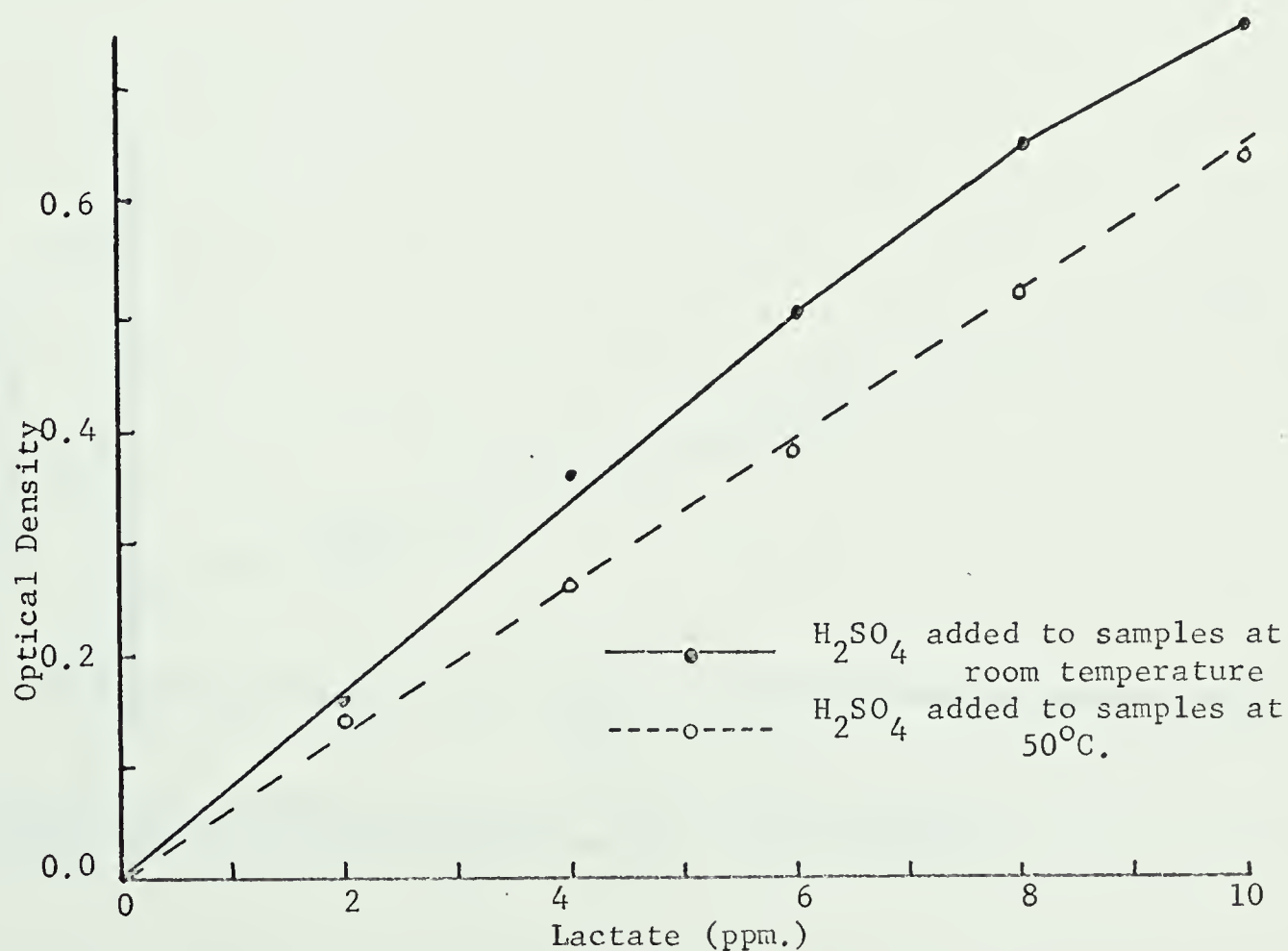


Figure 2(A3). Effect on the lactate standard curve of adding H₂SO₄ to samples incubated at different temperature in pure solution. ($\lambda = 570$ mμ.)

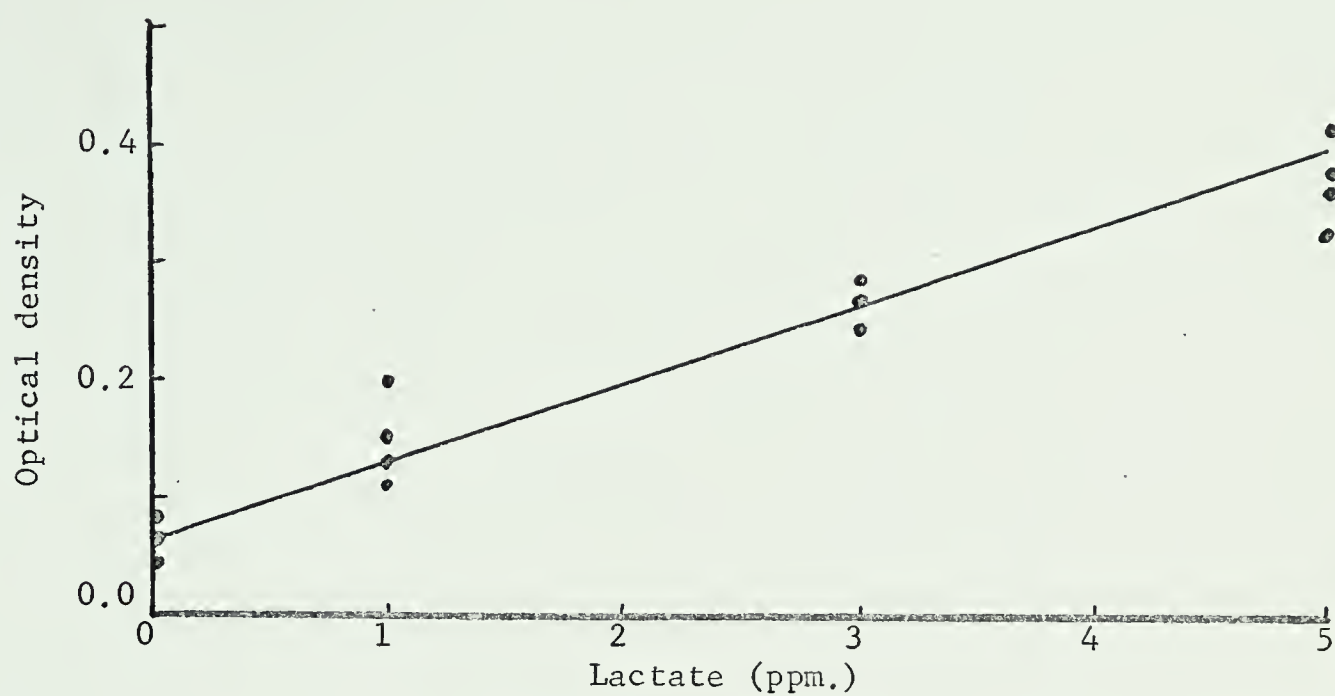


Figure 3(A3). Standard curve for lactate in the presence of cells of isolate 92 and B20 resting cell medium. ($\lambda = 570 \text{ m}\mu$.)

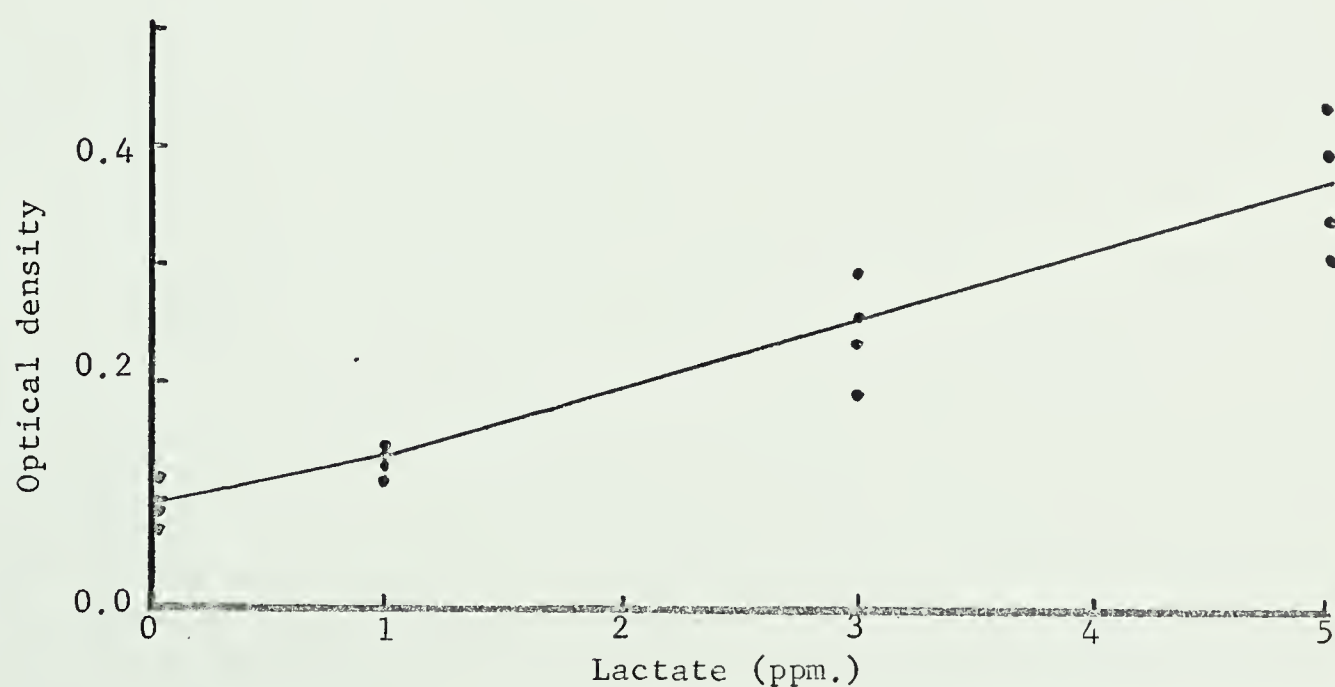


Figure 4(A3). Standard curve for lactate in the presence of cells of isolate 92 and A20 resting cell medium. ($\lambda = 570 \text{ m}\mu$.)

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